IMAGING DYNAMIC VOLUME CHANGES IN ASTROCYTES

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ABSTRACT

Astrocytes, the major type of non-neuronal cells in the brain, play an important functional role in the brain's extracellular potassium (K^+) and pH homeostasis. Pathological brain states have been shown to cause astrocyte swelling. However, these volume changes have never before been verified to occur in response to physiological activity. In the present thesis, two-photon laser scanning microscopy was used to visualize real-time astrocyte volume changes in the stratum radiatum of the CA1 region of the hippocampus. Astrocyte somas and primary processes were observed to swell by 19.0±0.9% in response to a physiological (3 mM) increase in the concentration of extracellular K^+. Astrocyte swelling was partially mediated by K+ influx through inwardly rectifying K^+ channels (K_{ir}), as their inhibition resulted in a significant decrease of the increased K^+ induced astrocyte swelling (13.9±0.9%). In addition, the bicarbonate ion (HCO_3^-) was found to play a significant role in the increased K+ induced astrocyte swelling. The astrocyte swelling was significantly decreased when the influx of HCO_3^- was decreased in 1) a HCO_3^- free extracellular solution (5.4±0.7%), 2) in the presence of an extracellular carbonic anhydrase inhibitor (11.4±0.6%), and 3) when the activity of the sodium-bicarbonate cotransporter (NBC) was blocked (8.3±0.7%) . Conversely, astrocytes were found to shrink by 7.7±0.5% in response to γ-Amino-butyric Acid (GABA) receptor activation. $GABA_A$ receptor mediated astrocyte shrinkage was significantly decreased to 5.0±0.6% when HCO_3^- efflux was reduced. Furthermore, in this thesis it was shown for the first time that astrocytes swell in response to neuronal stimulation (4.0±0.4%). This activity induced astrocyte swelling was significantly decreased to 1.5±0.2% in a HCO_3^- free extracellular solution. These astrocyte volume changes may have important implications for the regulation of brain activity under both physiological and pathological brain states.

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LIST OF ABBREVIATIONS

α-syn	α-syntrophin
3D	Three-dimensional
$[K^+]_o$	Concentration of extracellular potassium
ACSF	Artificial cerebrospinal fluid
ACTZ	Acetazolamide
ATP	Adenosine triphosphate
AQP	Aquaporin
Ba^{2+}	Barium
CA	Carbonic anhydrase
Ca^{2+}	Calcium
Cl^-	Chloride
CNS	Central nervous system
CO_2	Carbon dioxide
ddH_2O	Double distilled water
DIA	Depolarization induced alkalinization
DIDS	4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
DMSO	Dimethyl sulfoxide
ECS	Extracellular Space
fEPSP	Field excitatory post-synaptic potential
GABA	γ-Amino-butyric Acid
GFAP	Glial fibrillary acidic protein
GLUT1	Glucose Transporter 1
H^+	Hydrogen
H_2O	Water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCO_3^-	Bicarbonate
K^+	Potassium
K_{ir}	Inwardly rectifying potassium channel
mGluR	Metabotropic glutamate receptor
Na^+	Sodium

NBC	Sodium-bicarbonate cotransporter
NKCC	Sodium-potassium chloride cotransporter
SEM	Standard error of the mean
SR101	Sulforhodamine 101
STPP	1-(4-sulfamoylphenylethyl)-2,4,6-trimethyl-pyridinium perchlorate
TTX	Tetrodotoxin

CHAPTER 1

INTRODUCTION

1.1 Astrocytes

There are two major cell types in the central nervous system (CNS), neurons and glia. Neurons have traditionally been viewed as the main signaling units in the brain (Dierig, 1994), while glia, by contrast were viewed as support cells, responsible for ensuring optimal neuronal functioning by providing neurons with structural and metabolic support (Haydon, 2001). This view of glial cells is perhaps best illustrated by the fact that the name "glia" literally means glue in Greek, based on the initial belief that they served only as the mortar that holds the neurons of the brain together (Kettenmann and Ransom, 2005). In humans, it is estimated that one type of glia, the astrocytes, contribute up to 50% of the total brain volume and outnumber neurons by a ratio of 1.4:1 (Chen and Swanson, 2003; Nedergaard et al., 2003; Verkhratsky and Parpura, 2010). This number far exceeds that which is needed to provide simple metabolic support to neurons, suggesting a greater role for astrocytes than as the simple support cells in the brain (Nedergaard et al., 2003). Over a century after their discovery, astrocytes are now known to play an essential role in an incredibly diverse set of brain functions such as brain development, extracellular homeostasis, metabolism, structural support, and most recently brain signaling (Verkhratsky and Parpura, 2010; Wang and Bordey, 2008).

1.1.1 Definition and characteristics

Astrocytes were initially named by Ramón y Cajal (1909) for their star-shaped appearance when stained using his gold sublimate method (Kettenmann and Ransom, 2005). It is now known however, that astrocytes are more spongiform than stellate in morphology (Figure 1.1; Bushong et al., 2002). This aspect of astrocyte morphology became apparent only as new staining and imaging methods have emerged, allowing for the visualization of the numerous fine processes that branch off of each major process (Bushong et al., 2002; Nedergaard et al., 2003). Interestingly, astrocyte processes appear to overlap very little with the processes of adjacent astrocytes (Bushong et al., 2002). Each astrocyte occupies its own distinct three-dimensional (3D) spatial territory within which it can come in contact with hundreds of neuronal processes,

thousands of synapses, and the cerebrovasculature at any given time (Halassa et al., 2007). One potentially important implication of this is that small "functional islands" may be formed within the spatial territory of each individual astrocyte, and the activity of nearby synapses can be coordinated through modulation by a single astrocyte (Halassa et al., 2007).

Astrocytes are a diverse group of cells with both morphological and distributional differences (Nimmerjahn, 2009). Within the astrocytes, a number of different cell types exist; these include the protoplasmic astrocytes of the grey matter, the fibrous astrocytes of the white matter, the Bergmann glia of the cerebellum, and the Muller cells of the retina (Kettenmann and Ransom, 2005; Kimelberg, 2010). All mature astrocytes can be characterized by a set of common features. In addition to their characteristic fine processes that contact neurons and blood vessels, another primary characteristic of all astrocytes is that, unlike neurons, they display no electrical excitability but appear to signal via intracellular calcium (Ca^{2+}) rises instead (Cornell-Bell et al., 1990; Kimelberg, 2010; Perea et al., 2009). Another set of astrocyte characteristics, and one that has been invaluable towards their identification, is their expression of the astrocyte specific glial fibrillary acidic protein (GFAP), S100β, and their uptake of the astrocyte specific dye sulforhodamine 101 (SR101; Kimelberg, 2010; Ludwin et al., 1976; Nimmerjahn et al., 2004). Astrocytes are also characterized by a very negative membrane potential, a high degree of inter-connectivity via gap junctional channels, and the ability to clear substances such as potassium (K^+), glutamate, and γ-amino-butyric Acid (GABA) from the extracellular space (ECS; Kimelberg, 2010).

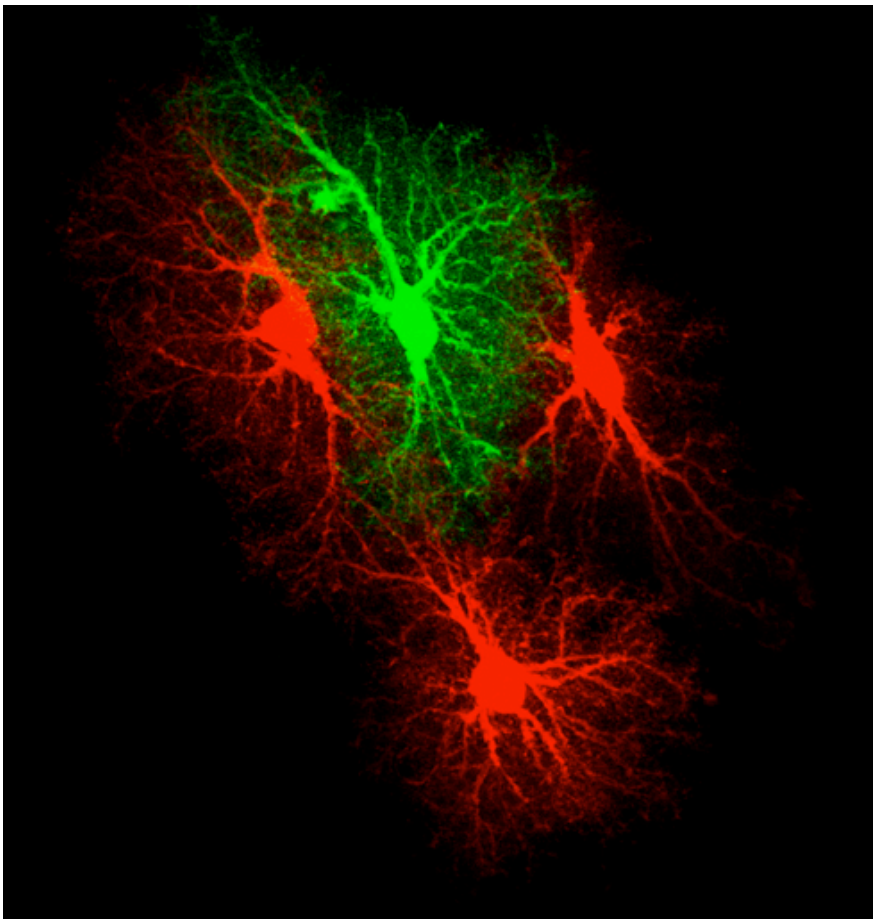

Figure 1.1. Hippocampal astrocytes occupy non-overlapping spatial domains.
High-magnification image of adjacent astrocytes in the CA1 region of the hippocampus. Optical slices were taken of neighbouring astrocytes that had been filled with either a green fluorescent dye (either Lucifer yellow or Alexa 488) or a red fluorescent dye (Alexa 586). Note the minimal region of interaction between the fine processes of adjacent astrocytes. Reproduced with permission from the Cell Centered Database.

1.1.2 Accepted astrocyte functions

Mature astrocytes serve many important functions in the brain. Several of their most widely studied and accepted functions are discussed in more detail below; these include providing metabolic support for neurons, neurotransmitter uptake and release, and extracellular K^+ and pH regulation.

1.1.2.1 Metabolic support

One of the first known astrocyte functions was the control of brain metabolism. Astrocyte endfeet surround close to 100% of the cerebrovasculature (Virgintino et al., 1997), placing them in the perfect anatomical position to mediate the movement of metabolic substrates such as glucose in and out of the brain. This glucose uptake is facilitated by the dense concentrations of GLUT1 glucose transporters in each endfoot (Kacem et al., 1998; Vannucci et al., 1997) that allow astrocytes to take up plasma glucose and make it available for use by the brain's cells (Wang and Bordey, 2008). It has been estimated that as much as 75% of glucose entering the brain does so through astrocytes (Hyder et al., 2006). Neurons have been hypothesized to obtain metabolic substrates such as lactate from astrocytes preferentially over glucose (Pellerin and Magistretti, 1994) but this is still a matter of much debate (see Schurr, 2006 for a detailed review of the controversy).

1.1.2.2 Neurotransmitter uptake and release

Up to 57% of synapses in the brain are surrounded by astrocyte endfeet (Ventura and Harris, 1999). The close association between astrocyte processes and neuronal pre and post-synaptic terminals, known as the tripartite synapse (Halassa and Haydon, 2010; Perea et al., 2009), facilitates bidirectional communication between astrocytes and neurons through the uptake and release of neurotransmitters (Haydon, 2001). Astrocytes are known to take up neurotransmitters such as glutamate and GABA from the ECS (Bergles and Jahr, 1998; Kinney and Spain, 2002; Roepstorff and Lambert, 1992). This uptake has several very important functional implications for the brain. Perhaps the most obvious of these functions is that in removing neurotransmitters from the ECS astrocytes may limit neurotransmitter spillover from the synapse, modulating synaptic plasticity and keeping them from accumulating to harmful levels (Kullmann and Asztely, 1998; Huang and Bordey, 2004). In particular, astrocyte

glutamate uptake appears to be critical for avoiding the build up of glutamate in the ECS that can lead to neuronal excitotoxicity (Anderson and Swanson, 2000; Rothstein et al., 1996; Tanaka et al., 1997; Zou et al., 2010). Once it has been taken up by the astrocyte, glutamate is recycled into its synaptically inactive precursor, glutamine, using the enzyme glutamine synthetase (Zou et al., 2010). The glutamine can then be passed back to neurons for reconversion into glutamate without affecting synaptic transmission (Hamilton and Attwell, 2010).

In addition to neurotransmitter uptake, astrocytes are also capable of neurotransmitter release (Parpura et al., 1994; Haydon, 2001). The release of neurotransmitters from astrocytes has been called gliotransmission, and includes the release of glutamate, adenosine-triphosphate (ATP), and D-serine (Halassa and Haydon, 2010; Hamilton and Atwell, 2010; Perea et al., 2009). Once released, these gliotransmitters can act directly on the extrasynaptic receptors of nearby neurons, making them active participants in the regulation of neuronal activity and synaptic transmission (Fellin and Haydon, 2005; Parpura et al., 1994; Perea et al., 2009).

1.1.2.3 Extracellular potassium regulation

Neuronal activity, through the firing of action potentials, leads to the release of K^+ into the ECS. Due to the limited volume of the ECS (Sykova, 2004a), even moderate amounts of neuronal activity can lead to a build up of extracellular K^+. A single action potential in a nerve can raise the concentration of extracellular K^+ ($[K^+]_o$) by as much as 1 mM (Ransom et al., 2000) and repetitive stimulation of a neuronal fiber pathway has been reported to raise the $[K^+]_o$ from the resting level of 3 mM to a ceiling level of 12 mM (Heinemann and Lux, 1977; Walz, 2000). Only under pathological conditions, such as ischemia and spreading depression, is this ceiling level of K^+ exceeded, and has been reported to reach up to 80 mM (Heinemann and Lux, 1977; Kofuji and Newman, 2004; Walz, 2000). Left unchecked, these increases in the $[K^+]_o$ can lead directly to neuronal hyperexcitability, increased synaptic transmission, and, in severe cases, seizures (Dichter et al., 1972; Wang and Bordey, 2008; Walz, 2000). Thus, for normal functioning to be maintained, K^+ must be tightly regulated in the brain at all times.

There are two main mechanisms by which astrocytes are proposed to contribute to K^+ homeostasis in the brain, both of which have gained significant experimental support; 1) K^+ spatial buffering and 2) K^+ uptake. The concept of K^+ spatial buffering, first proposed by Orkand et al. (1966), suggests that astrocytes take up K^+ from areas of high $[K^+]_o$ and redistribute it to

areas of lower $[K^+]_o$. The flow of K^+ is driven through the highly connected astrocyte network by a current loop that is generated by the difference in the network's membrane potential between the areas of high and low $[K^+]_o$ (Figure 1.2; Kofuji and Newman, 2004; Kimelberg, 2010; Orkand et al., 1966). The ability of astrocytes to buffer extracellular K^+ has been attributed to their high K^+ conductance. This conductance is due in large part to their expression of inwardly rectifying K^+ channels (K_{ir}s), which allow for K^+ to passively flow into the astrocyte (Higashi et al., 2001). Recently, through knockout studies, it has been demonstrated that K_{ir} channels are necessary for K^+ spatial buffering as well as for the maintenance of K^+ homeostasis in the brain (Djukic et al., 2007; Neusch et al., 2006; Olsen and Sontheimer, 2008). In the time since its proposal, the main evidence in support of the K^+ buffering hypothesis has come from electrophysiological and diffusion studies that have confirmed the existence of K^+ current loops and have found evidence to suggest trans-cellular K^+ movement in the brain, respectively (Gardner-Medwin and Nicholson, 1983; Kofuji and Newman, 2004; Nicolson et al., 1979; Walz, 2000). However, further experiments are needed to confirm that K^+ does in fact travel large distances through the astrocyte network, something that has proven difficult to demonstrate *in vivo* (Kimelberg, 2010).

Another simpler mechanism of astrocyte K^+ buffering, and one that is perhaps easier to demonstrate, is K^+ uptake. Whereas there is no net uptake of K^+ by astrocytes during K^+ spatial buffering, K^+ uptake involves a transient accumulation of K^+ within the astrocyte cytoplasm (Walz, 2000). For electroneutrality to be conserved, the uptake of K^+ must be accompanied by either a concurrent uptake of anions or efflux of a different type of cation (Kofuji and Newman, 2004). To account for this, the movement of a number of different ions has been proposed to occur along with the K^+ influx; namely the influx of Cl^- and/or the efflux of Na^+ (Ballanyi et al., 1987). Following neuronal activity, as the $[K^+]_o$ decreases, astrocytes release the excess K^+ back into the ECS and the resting K^+ equilibrium is restored (Figure 1.2; Kofuji and Newman, 2004; Walz, 2000). This transient accumulation of K^+ in the astrocyte cytoplasm has been demonstrated in both cell culture and brain slice preparations (Ballanyi et al., 1987; Walz et al., 1984), and is proposed to occur through several different mechanisms. The Na^+-K^+-ATPase, for example, has a low enough affinity for K^+ that it can be activated by the increased extracellular K^+ to transport K^+ into the cell (Simard and Nedergaard, 2004). Alternatively, the accumulation of extracellular K^+ may also create a driving force for passive K^+ uptake through K_{ir} channels or

for uptake through the Na-K-Cl cotransporters (NKCCs) (Kofuji and Newman, 2004; MacVicar et al., 2002; Simard and Nedergaard, 2004; Walz, 2000).

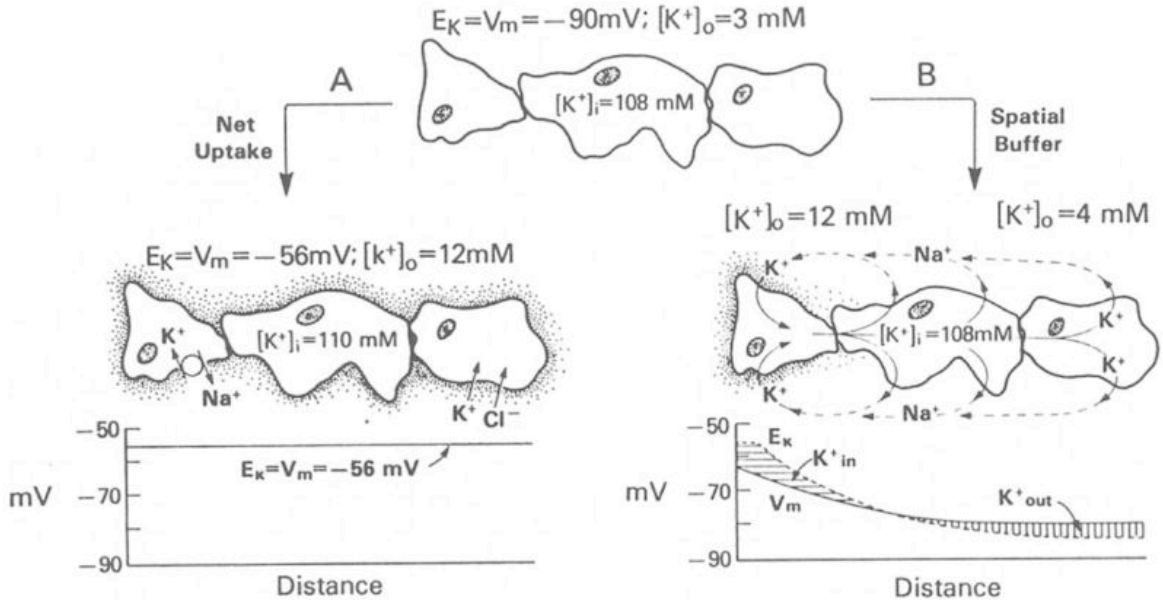

Figure 1.2. Extracellular K^+ regulation by astrocytes.
A diagram illustrating the role of astrocytes in extracellular K^+ homeostasis. Top: Three astrocytes coupled together via gap junctions have a resting membrane potential of approximately -90mV. **A** The mechanism of K^+ uptake. An increase in $[K^+]_o$ stimulates the net uptake of K^+ by astrocytes and a change in the membrane potential of the astrocyte network to approximately -56mV. **B** The mechanism of K^+ spatial buffering. An increase in $[K^+]_o$ creates a local depolarization of the astrocyte membrane. The difference in membrane potential between the areas of high and low $[K^+]_o$ creates a current loop that drives K^+ uptake in the regions of elevated $[K^+]_o$ and K^+ release in the regions of lower $[K^+]_o$. Reproduced with permission from Orkand et al., 1986.

1.1.2.4 pH regulation

The maintenance of normal pH in the brain is essential for proper cellular functioning. Alterations in pH can affect a wide range of cellular processes including neuronal excitability, synaptic transmission, metabolism, and the activity of many enzymes, channels, and transporters (Chesler and Kaila, 1992; Deitmer and Rose, 1996). Localized changes in the pH of the brain occur frequently, largely as a result of CO_2 extrusion from active neurons (Chesler and Kaila, 1992; Deitmer and Rose, 2010). Because of its small volume, the ability of the ECS alone to buffer these pH transients is limited; pH homeostasis in the CNS relies instead on the buffering capacity of the surrounding glia (Deitmer and Rose, 1996).

Astrocytes play an important role in the regulation of not only their own intracellular pH, but also the pH of the ECS (Brune et al., 1994, Chesler, 2003). The ability of astrocytes to respond to pH changes is due to their high expression of bicarbonate (HCO_3^-) transporters and carbonic anhydrase (CA) enzymes (Cahoy et al., 2008; Deitmer and Rose, 2010; Kimelberg, 2010). Astrocytes possess both cytosolic and extracellular, membrane-bound, forms of CA, which help determine the pH of a system by reversibly catalyzing the conversion of HCO_3^- and hydrogen (H^+) into carbon dioxide (CO_2) and water (H_2O) (Nagelhus et al., 2005; Obara et al., 2008; Tong et al., 2000) This reaction can occur in the absence of CA, but at a rate too slow to support the HCO_3^-/CO_2 buffering system (Deitmer and Rose, 1996). Once formed, HCO_3^- can be pumped through HCO_3^- transporters in either direction across the astrocyte membrane to influence both the astrocyte's intra and extracellular pHs (Deitmer and Rose, 2010; Brune et al., 1994).

In astrocytes, the primary HCO_3^- transporter involved in pH regulation is the sodium-bicarbonate cotransporter (NBC) (Deitmer and Rose, 2010). The NBC is activated by depolarization of the astrocyte membrane to pump sodium (Na^+) and HCO_3^- into the astrocyte, and by hyperpolarization to pump these same ions outwards (Deitmer and Rose, 2010). One main cause of astrocyte depolarization during neuronal activity, and as a result NBC activation, is the accumulation of K^+ in the ECS. As mentioned in the previous section, the uptake of K^+ in response to an increase in $[K^+]_o$ can cause a depolarization of the astrocyte membrane (Kofuji and Newman, 2004). Often, this K^+-induced depolarization is accompanied by an increase in the pH of the astrocyte cytoplasm that has been termed depolarization induced alkalinization (DIA; Deitmer and Rose, 1996; Pappas and Ransom, 1994). DIA is markedly reduced in astrocytes

that have been incubated in a HCO_3^- free extracellular solution (Pappas and Ransom, 1994) or with a NBC inhibitor (Deitmer and Rose, 1996), supporting the role of both the NBC and CA enzymes in K^+ induced astrocyte pH changes. An interesting implication of the link between K^+ and CO_2 clearance is that it may serve as a mechanism to couple the clearance of both an ionic and metabolic bi-product of neuronal activity, K^+ and CO_2 respectively, from the ECS at the same time (Nagelhus et al., 2004).

Neurotransmitters such as glutamate, GABA, and glycine have all been shown to induce pH shifts in the CNS (Obara et al., 2008). With respect to astrocyte pH regulation, of particular interest is the main inhibitory neurotransmitter, GABA, which has been shown to induce an acidification of the astrocyte cytoplasm and an alkalinization of the ECS (Kaila et al., 1991). This pH decrease is mediated by the activation of $GABA_A$ receptor-coupled chloride channels (Deitmer and Rose, 1996; Kaila and Voipio, 1987; Kaila et al., 1991) that, in astrocytes, allow for both chloride (Cl^-) and HCO_3^- efflux (Walz, 2002) with a suspected HCO_3^- to Cl^- permeability ratio of 0.2 based on studies performed in neurons (Staley et al., 1995). The GABA induced acidification is reduced when cells are incubated in either HCO_3^- free solution (Kaila et al., 1991) or with a CA inhibitor to block the formation of HCO_3^- (Pasternack et al., 1993).

1.2 Astrocyte volume changes

1.2.1 Glia have a high propensity for swelling

Cell volume changes occur primarily as a result of imbalances between the osmolarities of the intracellular and extracellular spaces that are generated by a variety of physiological and pathological conditions (Pasantes-Morales et al., 2000). Of the brain cells, glia are the most likely to undergo volume changes; swelling in response to electrical stimulation of the brain as well as ischemia, brain trauma and epilepsy (Holthoff and Witte, 1996; Lux et al., 1986; MacVicar and Hochman, 1991; Risher et al., 2009). This propensity for swelling been attributed in part to their high expression of aquaporin (AQP) water channels (Nagelhus et al., 2004; Nielsen et al., 1997). Neurons, by contrast, appear to lack functional water channels and are thus more resistant to volume changes (Andrew et al., 2007; Risher et al., 2009). Because glia are more likely to swell than neurons, indirect measurements of cellular volume, such as can be measured from changes in either the optical or diffusion properties of brain tissue, are often assumed to be due to glial swelling (Andrew and MacVicar, 1994; Holthoff and Witte, 1996;

Sykova et al., 2003). However, more recently, the relationship between cellular swelling and changes in the intrinsic optical properties of a tissue has been disputed in live preparations (Sykova et al., 2003), indicating that a more direct method is needed to measure glial swelling.

1.2.1.1 Aquaporin expression and colocalization with ion channels in glia

AQPs are the family of channels responsible for passive water conduction across cell membranes and are critical to maintain a balanced distribution of water between the intra and extracellular compartments of the brain (Andrew et al., 2007; Pasantes-Morales and Cruz-Rangel, 2010). There are three types of aquaporins expressed in the brain, AQP1, AQP4, and AQP9. Of these, AQP4 is the most widely expressed, being found predominantly in astrocytes and another type of brain glia called the ependymal cell (Amiry-Moghaddam and Ottersen, 2003; Nagelhus et al., 2004). Immunogold labeling of astrocyte AQP4 channels shows a distinct pattern of distribution that is guided by the presence of another protein, α-syntrophin (α-syn), in the membrane (Amiry-Moghaddam and Ottersen, 2003; Masaki et al., 2010; Nielsen et al., 1997). In particular, AQP4 expression appears to be the highest in astrocyte endfeet that come in contact with the pial surface, the cerebrovasculature, and glutamatergic synapses (Figure 1.3; Amiry-Moghaddam and Ottersen, 2003; Nagelhus et al., 2004). Due to their high expression of AQP channels, astrocytes have a high propensity to undergo rapid volume changes (Nagelhus et al., 2004). Furthermore, astrocyte AQPs strongly colocalized with K_{ir} channels. This colocalization may facilitate the concomitant uptake of water and K^+ when the $[K^+]_o$ has been increased (Masaki et al., 2010; Nagelhus et al., 1999; Nagelhus et al., 2004). When this colocalization is disrupted, as in α-syn$^{-/-}$ mice that lack functional AQP4 channels, K^+ clearance from the ECS is significantly decreased (Amiry-Moghaddam et al., 2003).

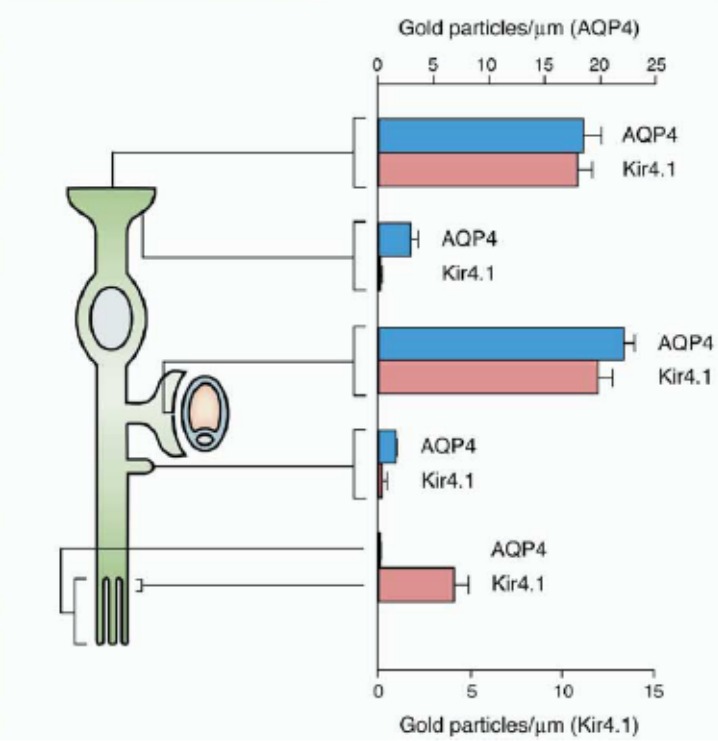

Figure 1.3. K_{ir} and AQP channel colocalization in the glial membrane.
A graph demonstrating the double immunogold labeling of K_{ir}4.1 and AQP4 in different membrane areas of astrocytes in the retina (Müller cells). The glial endfeet are enriched with both AQP4 and K_{ir}4.1. Note different scales for AQP4 (top) and K_{ir}4.1 (bottom). Reproduced with permission from Nagelhus et al., 2004.

1.2.2 Glial volume changes and the extracellular space

In the brain, the ECS forms a dynamic microenvironment surrounding the neurons and glia. The ECS is estimated to represent approximately 20% of the total brain volume, but its composition and size can change subject to physiological and pathological conditions (Nicholson and Sykova, 1998; Sykova, 2004b; Sykova and Chvatal, 2000). The most dramatic, and often irreversible, changes to the ECS occur as a result of morphological cell changes and the cell death that can occur in pathological states, such as ischemia and epilepsy, and through the natural process of aging (Sykova, 2004a, Sykova and Chvatal, 2000). However, smaller, reversible changes in the size and diffusion properties of the ECS do occur under physiological conditions as well, such as has been documented in response to neuronal activity (Dietzel et al., 1980; Ransom et al., 1985; Holthoff and Witte, 1996). For example, under experimental conditions neuronal activity can induce a shrinkage of up to 30% and an increase in the tortuosity (λ) of the ECS (Sykova et al., 2003; Ostby et al., 2009). This activity-induced ECS shrinkage appears to be closely linked to K^+ buffering and the movement of water that occurs during this process. As $K^+$is taken up by astrocytes (accompanied by a concurrent influx of anions to maintain electroneutrality), the intracellular osmolarity of the astrocytes increases. This osmotic gradient drives water influx into the astrocytes, causing astrocyte swelling and a local decrease in the volume of the ECS (MacVicar and Hochman, 1991; Ostby et al., 2009). Interestingly, in accordance with the hypothesis of K^+ spatial buffering, activity-induced ECS shrinkage is accompanied by an enlargement of the ECS at sites more distant from the activation, presumably in the area of K^+ release (Amiry-Moghaddam and Ottersen, 2003; Holthoff and Witte, 1996).

The diffusion of neuroactive substances through the volume of the ECS is an important signaling mechanism in the brain (Agnati et al., 1995; Chvatal et al., 2004; Sykova and Chvatal, 2000). Neuroactive substances, released from both neurons and astrocytes, diffuse through the ECS to distant extrasynaptic target receptors on both neurons and astrocytes. This type of signaling has been termed volume transmission (Agnati et al., 1995; Sykova, 2005; Sykova and Chvatal, 2000), and is the mechanism by which gliotransmission occurs (Theodosis et al., 2008). Unlike in the synapse, where signaling occurs between adjacent neurons, volume transmission facilitates signaling over much larger distances with the possibility of multiple target cells, including astrocytes (Sykova, 2005).

Volume transmission in the brain is greatly affected by the properties of the ECS such as the size of the ECS, cellular uptake, and the presence of cell membranes, fine processes, and the extracellular matrix (Kimelberg, 2000; Sykova, 2004b). As such, a change in either the shape or the volume of the ECS as a result of astrocyte swelling may affect not only the distance that signaling molecules can diffuse but also the pathways by which they can travel (Hrabetova, 2005; Kimelberg, 2000; Sykova et al., 1999). For example, one would expect that the swelling of an astrocyte process that directly surrounds a synapse might restrict neurotransmitter spillover from the synaptic cleft and limit the activation of extrasynaptic receptors (Nagelhus et al., 2004).

1.2.3 Principle mechanisms of astrocyte volume changes under physiological conditions

Astrocyte volume changes, identified primarily using cell culture and brain slice preparations, have been documented in response to hyper-, hypo- and iso-osmotic stimuli (Risher et al., 2009; Sykova et al., 2003; Walz, 1992; Walz, 2000). For the purpose of this study, only iso-osmotic swelling will be considered further.

Astrocytes respond to stimulation by both ions and neurotransmitters through the activation of various second messenger systems, ion channels, and transporters (Sykova and Chvatal, 2000). As a result of this activation, the osmolarity of the astrocyte may change as various ions diffuse or are pumped into and out of the cell and, when accompanied by water movement, can cause changes in the volume of the astrocytes surrounding the area of neuronal activity (often in the form of swelling) (Ostby et al., 2009; Sykova and Chvatal, 2000). Under physiological conditions the two primary activators of astrocyte swelling are extracellular glutamate and K^+, both of which increase in concentration in the ECS during neuronal activity (Simard and Nedergaard, 2004; McBain et al., 1990; Sykova et al., 2003).

The primary mechanism by which glutamate is believed to induce astrocyte swelling is through the activation of metabotropic glutamate receptors (mGluRs) (Bender et al., 1998, Hansson and Ronnback, 1992; Simard and Nedergaard, 2004). mGluRs are coupled to G-proteins and second messenger systems within the astrocyte which, when activated, can induce intracellular Ca^{2+} increases, the opening of K_{ir} channels, and changes to the astrocyte's metabolic processes (Hansson et al., 1994; Koyama et al., 1991; Vargova et al., 2001). Glutamate application has also been shown to activate astrocyte electrogenic Na^+-glutamate transporters, NKCCs, and the Na^+-K^+-ATPase pump, the activity of which could generate an osmotic gradient

large enough to induce swelling (Bender et al., 1998; Hansson et al., 1994; Schneider et al., 1992). However, glutamate application *in vivo* also results in an increase in the $[K^+]_o$ (Vargova et al., 2001). Thus, in addition to the above-mentioned mechanisms, glutamate-induced astrocyte swelling may be occurring, at least in part, as a result of extracellular K^+ accumulation (Kempski et al., 1991). Interestingly, although GABA, like glutamate, is known to induce pH shifts in the astrocyte cytoplasm, astrocyte volume changes in response to GABA application have never been observed (Hansson and Ronnback, 1992).

Astrocyte swelling in response to increased extracellular K^+ has been widely studied in both cell culture and hippocampal slice preparations. Through these studies, a variety of transporters and channels have been identified as of potential importance to astrocyte volume regulation, these include: the Na^+-K^+-ATPase pump, the NKCC1, the K_{ir}, the K^+-Cl^- cotransporter (KCC1), the NBC, the Cl^--HCO_3^- anion exchanger (AE), the Na^+ dependent Cl^--HCO_3^- exchanger (NDCBE) and the Na^+-H^+ exchangers (NHE) (Kettenmann and Ransom, 2004; Nagelhus et al., 2004; Otsby et al., 2009). These ion transporters are considered good candidates to account for the increase in astrocyte osmolarity because of their ability to actively take ions up into the cytoplasm (Otsby et al., 2009). From this list, it is apparent that many of the mechanisms believed to be responsible for the uptake of K^+ and other ions during K^+ induced astrocyte swelling are shared in common with the ionic mechanisms of K^+ buffering (see section 1.1.2.3, Extracellular potassium regulation).

The cotransporter that has been the most widely implicated in K^+ induced astrocyte swelling is the NKCC1. The NKCC1 actively transports one each of K^+ and Na^+ and two Cl^- ions into the astrocyte cytoplasm, generating an osmotic gradient capable of driving water influx (Payne et al., 2003). Inhibition of the NKCC1 cotransporter diminishes the prominent astrocyte swelling that is induced by increases in the $[K^+]_o$ of up to 50 mM or more (Juurlink et al., 1992; Su et al., 2002; Sykova et al., 1999). For example, an increase of 75 mM in the $[K^+]_o$ was shown to induce a swelling of 20.2 +/- 4.9% (Su et al., 2002). Using a similar increase in $[K^+]_o$ (57 mM), Walz (1991) observed an acidification of the astrocyte cytoplasm that occurred in conjunction with the swelling. Both the swelling and the acidification were diminished when the astrocytes are incubated in HCO_3^- free solution, indicating that the swelling may have some dependence on HCO_3^- (Walz, 1991) and the primary astrocyte HCO_3^- transporter, the NBC.

In an effort to provide a clearer explanation of the mechanisms underlying the phenomenon of ECS shrinkage due to astrocyte swelling, Otsby and colleagues (2009) performed a study using mathematical modeling. These authors found that the activity of either NKCC1 or NBC, in addition to passive ion transport mechanisms, appears to be necessary to account for astrocyte swelling on a scale that would produce a shrinkage of the ECS (Otsby et al., 2009). Passive ion uptake in combination with the activity of Na^+-K^+-ATPase and AQP water transport also appeared to be sufficient to generate the swelling, but only under extracellular K^+ levels far higher than those observed under normal physiological conditions (Otsby et al., 2009).

A major limitation of the currently available literature on K^+ induced astrocyte swelling is that the levels of extracellular K^+ used in these experiments are often much higher than the normal ceiling level of 12 mM (Heinemann and Lux, 1977; Walz, 2000). The limited number of studies that have investigated astrocyte swelling in response to smaller K^+ increases have found no effect (Juurlink et al., 1992, Sykova et al., 1999; Sykova et al., 2003). For example, Sykova and colleagues (2003) found that small increases in $[K^+]_o$ (6 or 10 mM) did not effect diffusion in the ECS, a finding that was interpreted as an indication that no cellular swelling had occurred (although it should be noted that they did find an increase in GFAP expression in these astrocytes). Indeed, only when the $[K^+]_o$ was increased to 50-80 mM were decreases in the volume of the ECS measured (Sykova et al., 2003).

However, it is possible that the methods employed to measure cell volume changes by the studies represented in the current literature may not have been sensitive enough to detect more subtle changes in volume, had there been any. As a result, the currently available literature reflects only what might be occurring during states such as ischemia, trauma, or status epilepticus (Kimelberg, 2000), leaving very little known about astrocyte swelling under physiological conditions.

CHAPTER 2

RATIONALE, HYPOTHESES AND OBJECTIVES

The aim of this thesis is to examine astrocyte volume changes in response to the physiological brain stimuli of increased $[K^+]_o$, $GABA_A$ receptor activation, and neuronal activity. Furthermore, the role K_{ir}s, glutamate, and HCO_3^- influx and efflux will be assessed as potential mediators of these volume changes.

Specific aims:

1) To observe the magnitude of astrocyte swelling induced by a physiological increase in $[K^+]_o$.

Astrocyte swelling in response to large, pathological increases in $[K^+]_o$ has been well established (Su et al., 2002; Sykova et al., 1999; Walz, 1991). However, at present, smaller increases in $[K^+]_o$ are not believed to induce significant astrocyte swelling (Sykova et al., 2003). This is at odds with the well studied phenomena of activity induced changes in the light transmittance and ECS of brain tissues, which have previously been attributed to astrocyte swelling (Holthoff and Witte, 1996; MacVicar and Hochman, 1991; Otsby et al., 2009; Ransom et al., 1985). In light of this discrepancy, the first objective of this thesis was to observe astrocyte swelling in response to a physiological increase in $[K^+]_o$. To evaluate the swelling, two-photon imaging will be used to obtain real-time 3D reconstructions from z-stacks of images taken of hippocampal astrocytes as they are exposed to a 3 mM increase in $[K^+]_o$.

Hypothesis: Using this more sensitive method of volume measurement, astrocyte swelling in response to the small increase in $[K^+]_o$ will be observed.

2) To investigate the mechanism by which K^+ induced swelling occurs.

For this, three separate mechanisms were considered:

a) Potassium uptake

Although widely speculated about, the mechanism by which K^+ induces astrocyte swelling remains largely unknown. Given the strong colocalization between K_{ir} channels and AQP4 channels, a coupling of water and K^+ influx is believed to underlie K^+ induced astrocyte swelling (Nagelhus et al., 2004). To examine the contribution of K_{ir} channels, the swelling

induced by a 3 mM increase in $[K^+]_o$ will be observed in hippocampal slices that have been pre-incubated with the K_{ir} channel blocker barium (Ba^{2+}).

Hypothesis: The application of Ba^{2+} to block K_{ir} channel activity will decrease the magnitude of the K^+ induced astrocyte swelling.

b) Glutamate release

Large increases in $[K^+]_o$ can lead directly to increased neuronal excitability and glutamate release (Wang and Bordey, 2008; Walz, 2000). Glutamate has previously been shown to induce astrocyte swelling (Bender et al., 1998; Hansson and Ronnback, 1992) and for this reason is a potential contributor to the K^+ induced astrocyte swelling. To examine the contribution of glutamate release on the swelling, hippocampal slices will be pre-incubated in the voltage-gated Na^+ channel blocker tetrodotoxin (TTX) to inhibit neuronal activity, and therefore the release of glutamate.

Hypothesis: Any increase in glutamate release as a result of the 3 mM increase in $[K^+]_o$ will be too small to have a significant effect on the magnitude of the swelling.

c) Bicarbonate

The release of K^+ from active neurons induces a rapid alkalinization of nearby astrocytes (Pappas and Ransom, 1994; Deitmer and Rose, 1996). This alkalinization is markedly decreased with the removal of HCO_3^- from the bath solution and by the application of the NBC inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (Brookes an Turner, 1994; Chesler and Kraig, 1987; Pappas and Ransom, 1994). A role for HCO_3^- in astrocyte swelling has been hypothesized (Walz, 1991), but has only been observed in cultured astrocytes that have been exposed to pathologically high $[K^+]_o$. To investigate the role the HCO_3^- plays in K^+-induced swelling, the magnitude of the swelling induced by a 3 mM increase in $[K^+]_o$ will be observed under conditions of decreased HCO_3^- influx. Three different methods will be used to decrease HCO_3^- influx: the incubation of brain slices in a HCO_3^- free extracellular solution, the inhibition of the formation of extracellular HCO_3^- using a cell impermeant CA inhibitor, and finally, the application of DIDS to inhibit the HCO_3^- influx.

Hypothesis: All three methods used to decrease HCO_3^- influx will significantly decrease the magnitude of the astrocyte swelling that occurs in response to a 3 mM increase in $[K^+]_o$.

3) To observe whether $GABA_A$ receptor activation induces astrocyte shrinkage in a HCO_3^- dependent manner.

The application of muscimol, a $GABA_A$ receptor agonist, results in an acidification of the intracellular pH of astrocytes, the opposite of what is seen in response to increases in extracellular K^+ (Kaila et al., 1991; Chen and Chesler, 1992). However, studies conducted on cultured astrocytes have found that $GABA_A$ receptor activation has no significant effect on astrocyte volume (Hansson and Ronnback, 1992). Given that HCO_3^- influx has been linked to astrocyte swelling, this thesis next aims to investigate astrocyte volume changes in response to $GABA_A$ receptor activation using the more sensitive two-photon imaging method to detect volume changes. In addition, the dependence of these volume changes on HCO_3^- will be assessed using a membrane permeant CA inhibitor to block the formation of intracellular HCO_3^-, and thereby decrease HCO_3^- efflux.

Hypothesis: The activation of $GABA_A$ receptors will induce a decrease in the volume of astrocytes in a manner that is dependent on HCO_3^- efflux.

4) To observe whether neuronal activity is sufficient to induce astrocyte volume changes.

Astrocytes are the brain cells with the highest propensity to swell (MacVicar and Hochman, 1991; Risher et al., 2009). For this reason, the changes in the optical and ECS properties that are observed with neuronal activation have often been attributed to astrocyte swelling (MacVicar and Hochman, 1991, Otsby et al., 2009; Sykova et al., 2003). However, real-time astrocyte volume changes in response to neuronal stimulation have never been measured directly. To observe whether neuronal activity was sufficient to induce astrocyte volume changes, astrocytes will be imaged from within an area receiving direct neuronal stimulation. This experiment will be repeated on astrocytes from brain slices that are incubated in HCO_3^- free ACSF to further confirm the role of HCO_3^- in astrocyte swelling.

Hypothesis: Astrocytes imaged from within the area of neuronal activity will swell in a manner that is dependent on HCO_3^- influx.

CHAPTER 3

MATERIALS AND METHODS

3.1 Hippocampal slice preparation and solutions

Hippocampal slices were prepared from male Sprague Dawley rats, aged 17-22 days postnatal, in accordance with procedures approved by the University of Saskatchewan Animal Care Committee. Rats were anesthetized with halothane, decapitated, and the brains rapidly removed. Horizontal hippocampal slices (400 μm thick) were cut using a vibrating microtome (VT1200, Leica Microsystems, Nussloch, Germany) into ice-cold (0-4°C) sucrose cutting solution containing (in mM): 87 NaCl, 75 Sucrose, 2.5 KCl, 25 $NaHCO_3$, 10 Glucose, 7 $MgCl_2$, and 0.5 $CaCl_2$ oxygenated with 95% O_2 - 5% CO_2. Slices were then incubated in artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 10 Glucose, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 2 $CaCl_2$ at 32°C for 30 minutes and then room temperature until use. Following a minimum of 30 minutes equilibration time, slices were transferred to a submerged recording chamber where they were constantly perfused with oxygenated ACSF at a rate of 1-2 mL/min and maintained at a temperature of 24±1°C using an inline solution heater (Warner Instruments).

For HCO_3^- free experiments, immediately after sectioning slices were transferred into 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered ASCF containing 130 NaCl, 2.5 KCl, 25 HEPES, 10 Glucose, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 2 $CaCl_2$, bubbled with 100% O_2 and buffered to a pH of 7.4 with NaOH. The high K^+, HCO_3^- and HEPES buffered, solutions were prepared by adding 3 mM KCl to the original solution recipes and removing an equimolar amount of NaCl. During an experiment the hippocampal slice was switched from low to high K^+ solutions through a system of manual valves, this allowed the solutions to be switched without affecting the rate of perfusion in the recording chamber.

3.2 SR101 dye loading

Astrocytes were identified in the stratum radiatum of the hippocampus via the labeling of the astrocyte specific dye SR101 (Sigma; Nimmerjahn, 2004). Hippocampal slices were incubated for 15 minutes in a solution of 25-100 μM SR101 dissolved in ACSF. To remove any

non-specific labeling, after dye application the slices were perfused continuously with ACSF for 2 hours before imaging.

3.3 Field potential stimulation and recording

Field excitatory postsynaptic potentials (fEPSPs) were evoked through electrical stimulation of the Schaffer collateral fibers in the CA3 region as a means of confirming that neuronal activity was occurring within the imaged area. Stimulation was performed using a Master-8-cp stimulator (A.M.P.I, Jerusalem, Israel) coupled to an ISO-Flex stimulus isolator (A.M.P.I, Jerusalem, Israel). Current pulses were delivered through a bipolar tungsten electrode (FHC, ME) in 200 μsec pulses at an intensity that was large enough to evoke a population spike, but <40V. fEPSPs were recorded from the CA1 region of the hippocampus using glass microelectrodes filled with ACSF (resistance of 3-5 MΩ). Recorded signals were amplified using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and were digitized using a Digidata 1440 (Molecular Devices, Sunnyvale, CA). Prior to the beginning of each experiment, a stable baseline was established for no less than 30 minutes using test pulses delivered at 0.02 Hz.

3.4 Two-photon imaging

SR101 loaded astrocytes in the stratum radiatum of the CA1 region of the hippocampus were imaged a minimum of 50 μm below the surface of the slice using a two-photon laser-scanning microscope. The Mai Tai *XF* Ti:Sapphire laser source (Spectra-Physics, Irvine, CA) was coupled to a custom modified Olympus BX51WIF microscope equipped with a Prairie Ultima scanhead using a 36 μs dwell time (Prairie Technologies Inc., Middleton, WI) and a 40X/0.8 numerical aperture water immersion objective lens. The SR101 fluorophore was excited at a wavelength of 835 nm and the resulting epifluorescence was detected with external detectors fitted with a red bandpass emission filter (607/45 nm). To minimize photobleaching and ensure astrocyte health, the laser intensity used for imaging was kept to a minimum. Astrocytes within the stratum radiatum of CA1 region of the hippocampus were clearly identified by their bright SR101 labelling and their characteristic morphology (Figure 3.1). To quantify astrocyte volume changes, two-photon z-stack images using an x-y spatial resolution of 0.29 μm/pixel were taken

in 1 μm z-steps using the software program Prairie-view (Prairie Technologies Inc., Middleton, WI).

To determine the time course of the astrocyte volume changes, z-stacks were taken at 10-minute intervals to a maximum of 40 minutes. For these experiments, each z-stack typically contained 5-7 astrocytes and lasted an average of 2-3 minutes. For the neuronal stimulation experiments astrocytes were imaged from directly in front of the recording pipette to ensure that they were within the area of neuronal activity (Figure 3.2). Three control z-stacks (30 seconds each) were imaged at 1 min intervals prior to stimulation, followed immediately by a continuous set of z-stacks (30 seconds each) imaged during the stimulation. To ensure careful quantification each z-stack contained an average of only two astrocytes.

3.5 Image analysis and volume measurements

Z-stacks were compressed into a single, two-dimensional maximum intensity projection using the software program Image J (Figure 3.1). The maximum intensity projections at each imaging time-point were overlayed, aligned, and cropped to contain only the soma and primary processes of individual astrocytes using Adobe Photoshop CS3 software. This process ensured that each cell was cropped identically at each time-point throughout the experiment. To clearly define individual astrocyte somas and primary processes, the images corresponding to individual astrocytes were imported into Image J where the fluorescence from the background and fine processes was removed using the threshold function. For individual cells, each time-point was thresholded at the same level. The area of the astrocyte was calculated by Image J as a percentage of the total image area, this eliminated any measuring bias that may be created by manually tracing or outlining cells. The percent change in the area was calculated and interpreted as an indication of a change in the total volume of the astrocyte.

Prior to any analysis the health of each hippocampal slice was determined based on cell morphology and the quality of the SR101 dye loading. All trials where the cells were not clearly defined or where the dye intensity was not stable for the duration of the experiment were discarded.

3.6 Drug application

All chemicals were bath applied through direct addition to the perfusate. The Ba^{2+}, purchased from Sigma-Aldrich (St. Louis, MO), was dissolved in ddH_2O and applied at a concentration of 200 μM (D'Ambrosio et al., 2002) a minimum of 30 minutes before imaging. The TTX, purchased from Alomone Labs (Jerusalem, Israel), was dissolved in ddH_2O and was applied at a concentration of 1 μM a minimum of 15 minutes before imaging. The membrane impermeant CA inhibitor 1-(4-sulfamoylphenylethyl)-2,4,6-trimethyl-pyridinium perchlorate (STPP), received as a gift from Claudio Supuran (University of Florence, Italy), was dissolved in dimethyl sulfoxide (DMSO) and applied at a concentration of 100 μM (based on personal communication with Claudio Supuran) for a minimum of 30 minutes before imaging. The 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), purchased from Sigma-Aldrich (St. Louis, MO), was dissolved in DMSO and was applied at a concentration of 500 μM for a minimum of 30 minutes before imaging. The muscimol, purchased from Tocris Bioscience (Ellisville, MO), was dissolved in ddH_2O and was applied at a concentration of 20 μM (Kaila et al., 1991) and was applied immediately after the first Z-stack was acquired. The acetazolamide, purchased from Sigma-Aldrich (St. Louis, MO), was dissolved in DMSO and applied at a concentration of 100 μM (Pasternack et al., 1993) for a minimum of 30 minutes prior to the application of muscimol. Care was taken to ensure that the concentration of DMSO in the final solution never exceeded 0.1%.

3.7 Statistics

Significant differences between population means were assessed using either a Student's t-test or a one-way ANOVA followed by a Bonferroni post-hoc analysis, where appropriate, with a confidence level of $p<0.05$. The effects of all treatments on astrocyte swelling or shrinking were evaluated by comparing their respective interleaved control and treatment slices. Results are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using the software program PASW Statistics version 18.0.

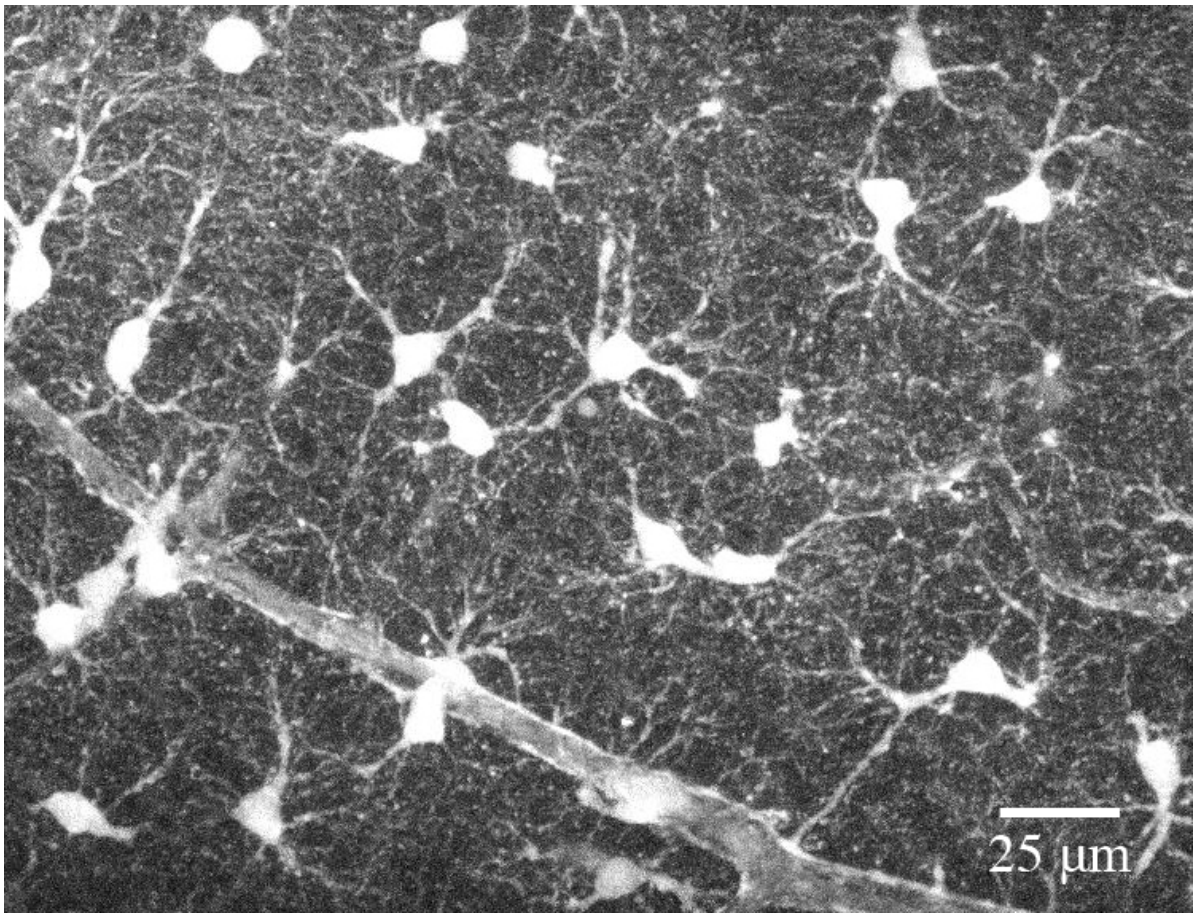

Figure 3.1. Maximum intensity projection of hippocampal astrocytes.
Astrocytes imaged in the stratum radiatum of the CA1 region of the hippocampus were selectively loaded using the astrocyte specific dye SR101 and identified based on their bright labeling and classic astrocyte morphology. Two-photon z-stacks, taken of the astrocytes in 1 μm steps, were compressed into a single, two-dimensional maximum intensity projection for analysis.

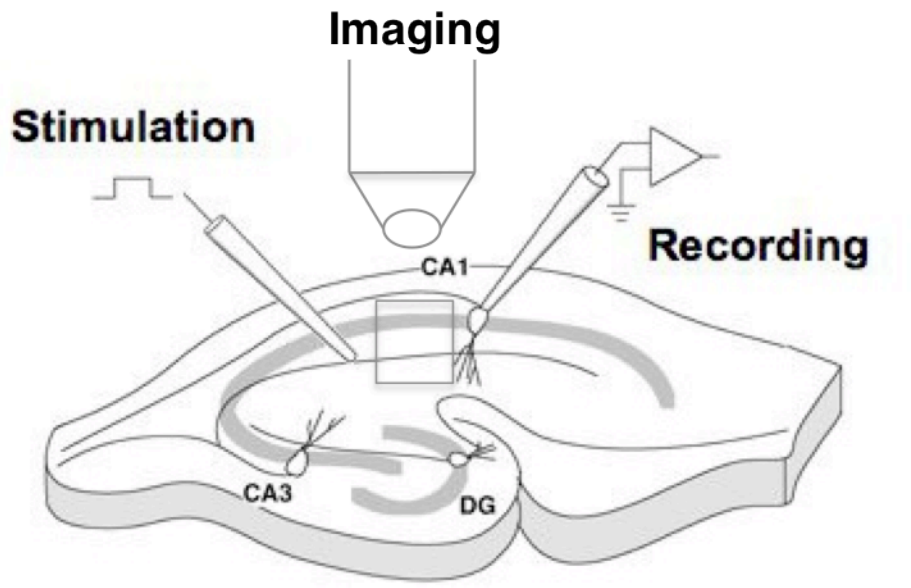

Figure 3.2. Schematic drawing of a hippocampal slice showing the experimental setup used during neuronal stimulation trials.
A bipolar stimulating electrode was used to electrically stimulate the Schaffer collateral fiber pathway. Field potentials were recorded from the stratum radiatum of the CA1 region in hippocampus using a glass recording pipette. Two-photon z-stacks were taken of astrocytes located directly in from of the recording electrode to ensure they were within the area of neuronal activation.

CHAPTER 4

RESULTS

4.1 Astrocyte swelling in response to physiological increases in extracellular K^+

A 3 mM increase in $[K^+]_o$ was used to determine whether astrocyte swelling occurs in the stratum radiatum of the CA1 region of the hippocampus during a modest physiological increase in $[K^+]_o$.

Two-photon z-stack images of astrocytes were taken to establish an initial volume from hippocampal slices incubated in ACSF containing 2.5 mM extracellular K^+. The slices were then perfused with ACSF containing 5.5 mM extracellular K^+ and series of z-stack images were taken of the astrocytes at 10 minute intervals to establish the time-course of astrocyte volume changes. The average astrocyte area was found to be 113.0 ± 2.1μm^2 (n=314 from 57 slices) before any treatment was applied. Astrocytes were observed to swell by 9.3±0.7% at the 10 minute time point, 16.1±1.1% at the 20 minute time point, 18.1±1.0% at the 30 minute time point, and 19.0± 0.9% at the 40 minute time point. The magnitude of the astrocyte swelling was found to reach a maximum at approximately 40 minutes during perfusion with increased $[K^+]_o$ ACSF (Figure 4.1 D). The 3 mM increase of $[K^+]_o$ induced a significant increase in the volume of the astrocyte somas and primary processes (19.0±0.9%, n=24 astrocytes from 6 brain slices; Figure 4.1) in comparison to astrocytes that were imaged over the same 40 minute experimental time period while remaining in the 2.5 mM K^+ control ACSF; -0.9±0.2% (n= 24 astrocytes from 4 slices); [$t(46)=22.094$, $p<0.05$; Figure 4.1E].

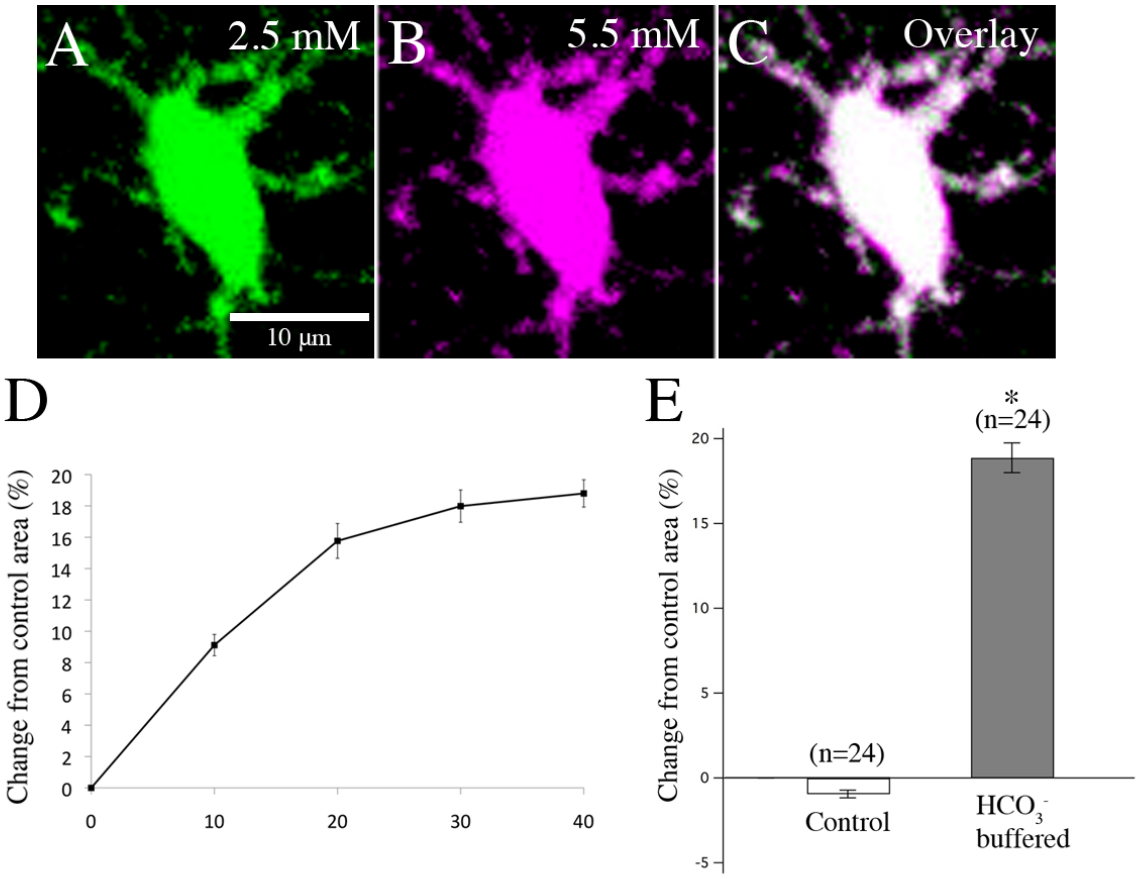

Figure 4.1. Astrocytes swell in response to a 3 mM increase in $[K^+]_o$.
A-B An image sequence showing astrocytes before (**A**) and after (**B**) a 3 mM increase in $[K^+]_o$. **C** An overlay of the astrocyte in 2.5 mM extracellular K^+ (**A**; green) and after the swelling in 5.5 mM extracellular K^+ (**B**; magenta) showing the volume differences under these conditions in magenta. **D** The time course of the astrocyte swelling, the magnitude of which plateaus over a 40-minute experimental period. **E** Bar graph displaying the changes in the area of the astrocyte somas and primary processes in control (2.5 mM $[K^+]_o$) ACSF (no fill) and 3 mM increase in $[K^+]_o$ (5.5mM $[K^+]_o$) ACSF (shaded) are shown as a percent change from the control area. Error bars denote the SEM.
* Statistical significance assessed using a Student's t-test, $p<0.05$.

4.2 Mechanisms of K^+ induced astrocyte swelling

4.2.1 Inhibition of K_{ir} channels reduces K^+ induced astrocyte swelling

To determine if K_{ir} channels play a role in K^+ induced astrocyte swelling, K^+ influx through K_{ir} channels was blocked using Ba^{2+}, a known K_{ir} channel inhibitor (Olsen and Sontheimer, 2008). Hippocampal slices were perfused with ACSF containing Ba^{2+} (200 μM) 30 minutes prior to imaging and throughout the course of the experiment. Ba^{2+} was found to significantly decrease the 5.5 mM $[K^+]_o$ induced swelling of the astrocyte somas and primary processes; 13.9±0.9% (n=34 astrocytes from 5 slices); [$t(56)=3.869$, $p<0.05$; Figure 4.2].

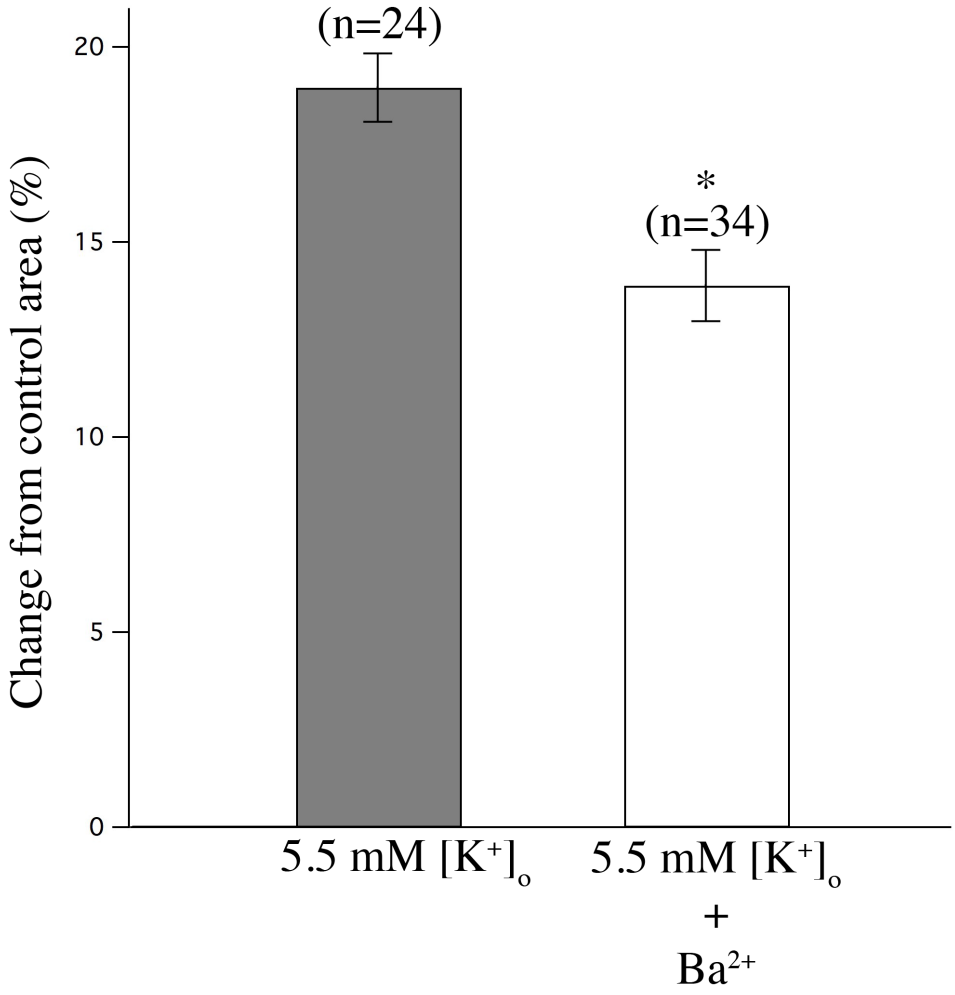

Figure 4.2. The effect of Ba^{2+} on K^+ induced astrocyte swelling.
5.5mM $[K^+]_o$ induced astrocyte swelling is significantly reduced in the presence of Ba^{2+}. Bar graph shows the percent changes in the area of the astrocyte somas and primary processes in 5.5 mM $[K^+]_o$ in control ACSF (shaded) and 5.5mM $[K^+]_o$ in the presence of Ba^{2+} (no fill). Error bars denote the SEM.
* Statistical significance assessed using a Student's t-test, $p<0.05$.

4.2.2 Increased neuronal excitability contributes minimally to increased $[K^+]_o$ induced astrocyte swelling

Experiments were performed in the presence of the voltage-gated Na^+ channel blocker TTX to determine if an increase in neuronal excitability played a role in the 5.5mM K^+ induced astrocyte swelling.

In ACSF containing TTX, a 3 mM increase in $[K^+]_o$ resulted in significantly less astrocyte swelling than was observed in response to the same increase in control ACSF; 16.4± 0.7% (n=30 astrocytes from 6 slices); [$t(52)=2.17$, $p=0.034$; Figure 4.3].

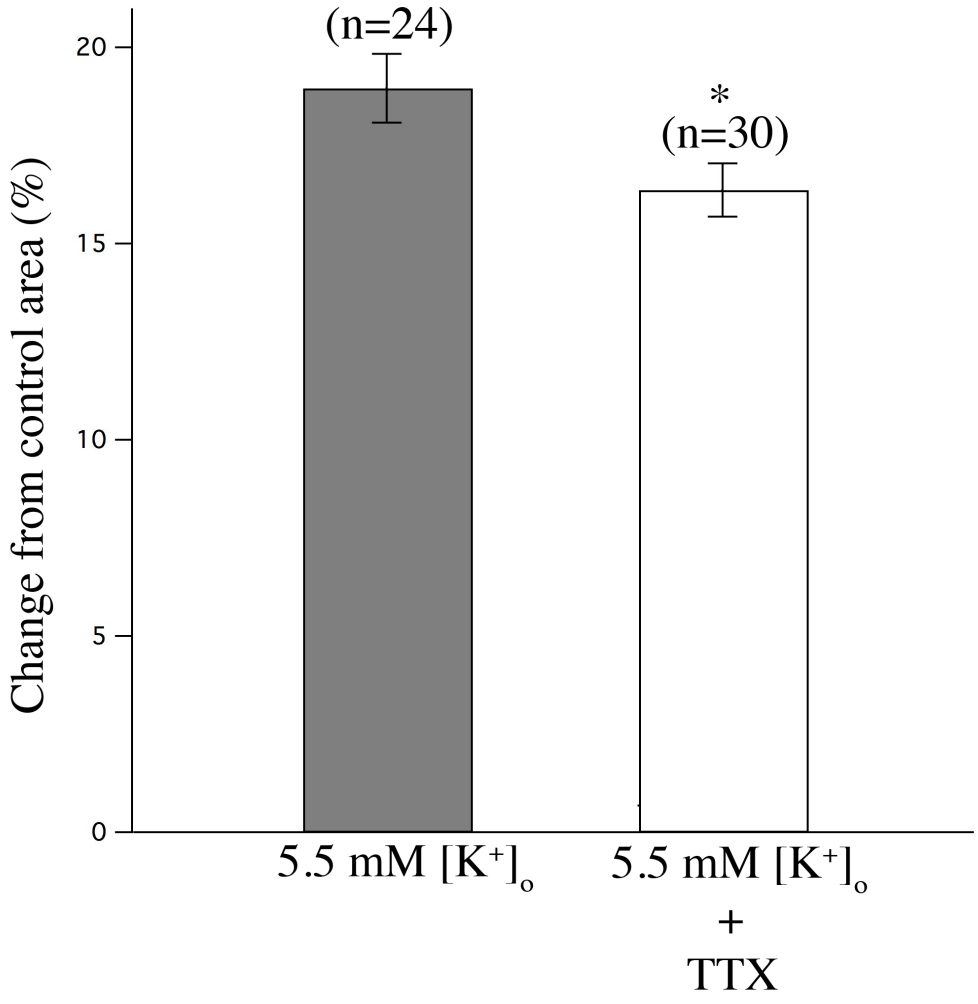

Figure 4.3. Astrocyte swelling occurs in ACSF containing TTX.
5.5mM $[K^+]_o$ induced astrocyte swelling is not significantly reduced in the presence of TTX. Bar graph shows the percent changes in the area of the astrocyte somas and primary processes in 5.5 mM $[K^+]_o$ in control ACSF (shaded) and 5.5mM $[K^+]_o$ in the presence of TTX (no fill). Error bars denote the SEM.
* Statistical significance assessed using a Student's t-test, $p<0.05$.

4.2.3 Increased $[K^+]_o$ induced astrocyte swelling is dependent on HCO_3^- influx

To investigate the role of HCO_3^- in the increased K^+ induced astrocyte swelling, the magnitude of the swelling was observed under three different conditions that were expected to reduce HCO_3^- influx. In the first two series of experiments, the levels of extracellular HCO_3^- were reduced either by incubating astrocytes in a HCO_3^- free ACSF, or by reducing the production of extracellular HCO_3^- using a cell impermeant CA inhibitor. A third set of experiments was performed to block HCO_3^- influx directly through the NBC cotransporter. A one-way ANOVA was conducted and revealed a significant effect when HCO_3^- influx was inhibited [$F(3,141)=49.80$, $p<0.05$].

Post hoc analysis revealed that incubating the hippocampal slices in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered HCO_3^- free ACSF significantly decreased the 5.5mM $[K^+]_o$ induced astrocyte swelling compared to the swelling observed under control, HCO_3^- buffered ACSF, conditions; 5.4±0.7% (n=31 astrocytes from 7 slices); [$p<0.05$; Figure 4.4].

The membrane impermeant CA inhibitor 1-(4-sulfamoylphenylethyl)-2,4,6-trimethyl-pyridinium perchlorate (STPP) (100 μM) was used to selectively inhibit the formation HCO_3^- in the extracellular solution. Post hoc analysis revealed that the 5.5mM $[K^+]_o$ induced astrocyte swelling was significantly reduced in the presence of STPP compared to the 5.5mM $[K^+]_o$ induced astrocyte swelling in control ACSF; 11.4±0.6% (n=50 cells from 7 slices); [$p<0.05$; Figure 4.4].

To examine the role HCO_3^- influx through the NBC plays during the increased K^+ induced swelling, the activity of the NBC was blocked with the NBC inhibitor DIDS (500 μM). Post hoc analysis revealed that NBC inhibition significantly decreased the 5.5mM $[K^+]_o$ induced astrocyte swelling compared to the astrocyte swelling observed in 5.5mM $[K^+]_o$ control ACSF; 8.3± 0.7% (n=40 astrocyte from 6 slices) [$p<0.05$; Figure 4.4].

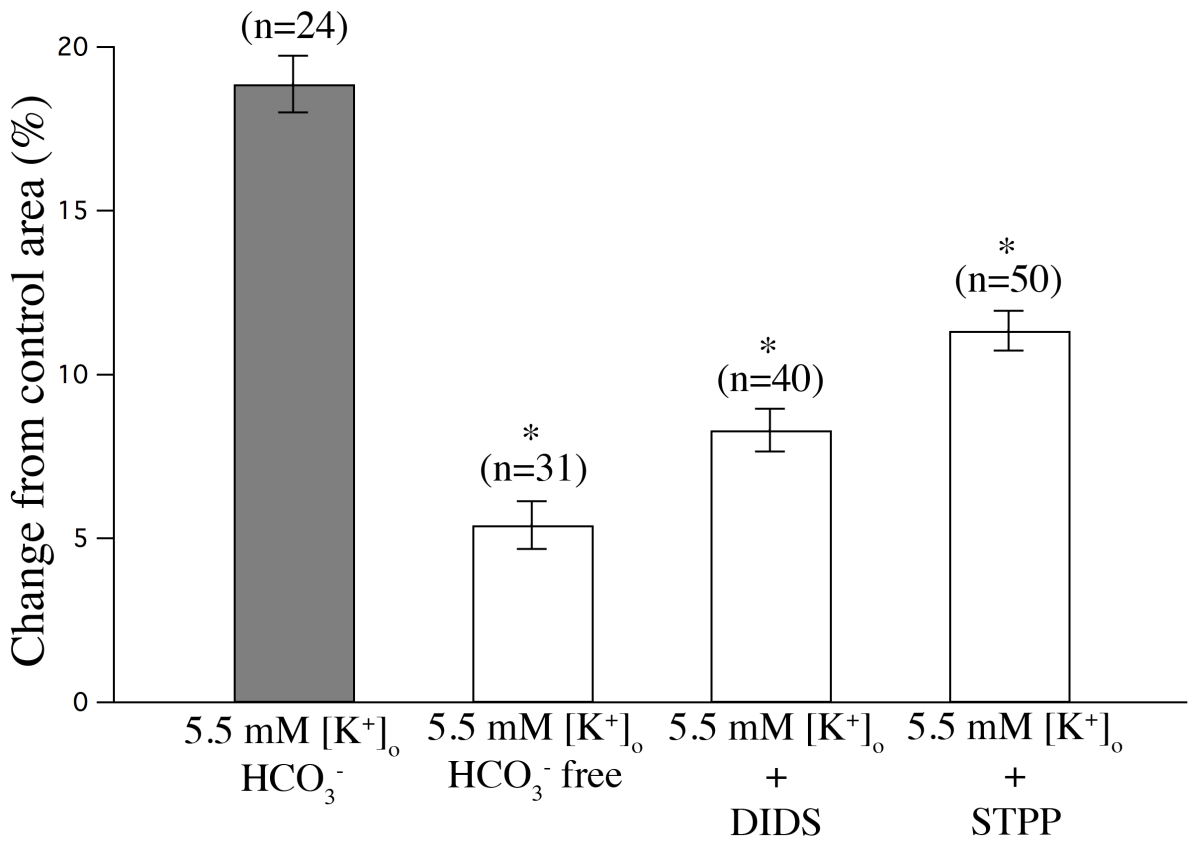

Figure 4.4. Increased K^+ induced astrocyte swelling is decreased in conditions of reduced HCO_3^- influx.

5.5mM $[K^+]_o$ induced astrocyte swelling is significantly decreased when the influx of HCO_3^- is reduced by incubating the astrocytes in HCO_3^- free ACSF, in the presence of the NBC blocker DIDS, and in the presence of the cell impermeant CA inhibitor STTP. Bar graph shows the percent changes in the area of the astrocyte somas and primary processes in 5.5 mM $[K^+]_o$ in HCO_3^- buffered ACSF (shaded) and 5.5mM $[K^+]_o$ in HCO_3^- free ACSF, with DIDS, and with STPP (all no fill). Error bars denote the SEM.

* Statistical significance assessed using a one-way ANOVA followed by a Bonferroni post hoc analysis, $p<0.05$.

4.3 Astrocyte shrinkage in response to $GABA_A$ receptor activation is dependent on HCO_3^- efflux

To determine whether $GABA_A$ receptor activation induces astrocyte volume changes, the volume of astrocytes was monitored in response to the application of the selective $GABA_A$ receptor agonist muscimol (20μM). $GABA_A$ receptor activation was found to cause a significant shrinking of the astrocyte somas and primary processes; 7.7± 0.5% (n=42 astrocyte from 8 slices); ($p<0.05$; Figure 4.5).

To determine if HCO_3^- plays a role in the $GABA_A$ induced astrocyte shrinkage via efflux through the $GABA_A$ receptor coupled channels, the membrane-permeant CA inhibitor acetazolamide (ACTZ) (100 μM) was used to block the formation of intracellular HCO_3^- during muscimol applications. Inhibition of intracellular HCO_3^- by ACTZ significantly decreased the amount of $GABA_A$ receptor mediated astrocyte shrinkage by muscimol; 5.0± 0.6% (n=34 astrocytes from 7 slices); ($p<0.05$; Figure 4.5). A Student's t-test confirmed that there is no significant difference between the shrinkage observed in control astrocytes that were incubated in 2.5 mM $[K^+]_o$ for 40 minutes and astrocyte that were incubated with ACSF containing ACTZ [$t(4.57)=1.45$, $p=0.212$].

A one-way ANOVA confirmed significance between the control, muscimol, and muscimol with ACTZ groups ($F(2,97)=46.28$, $p<0.05$).

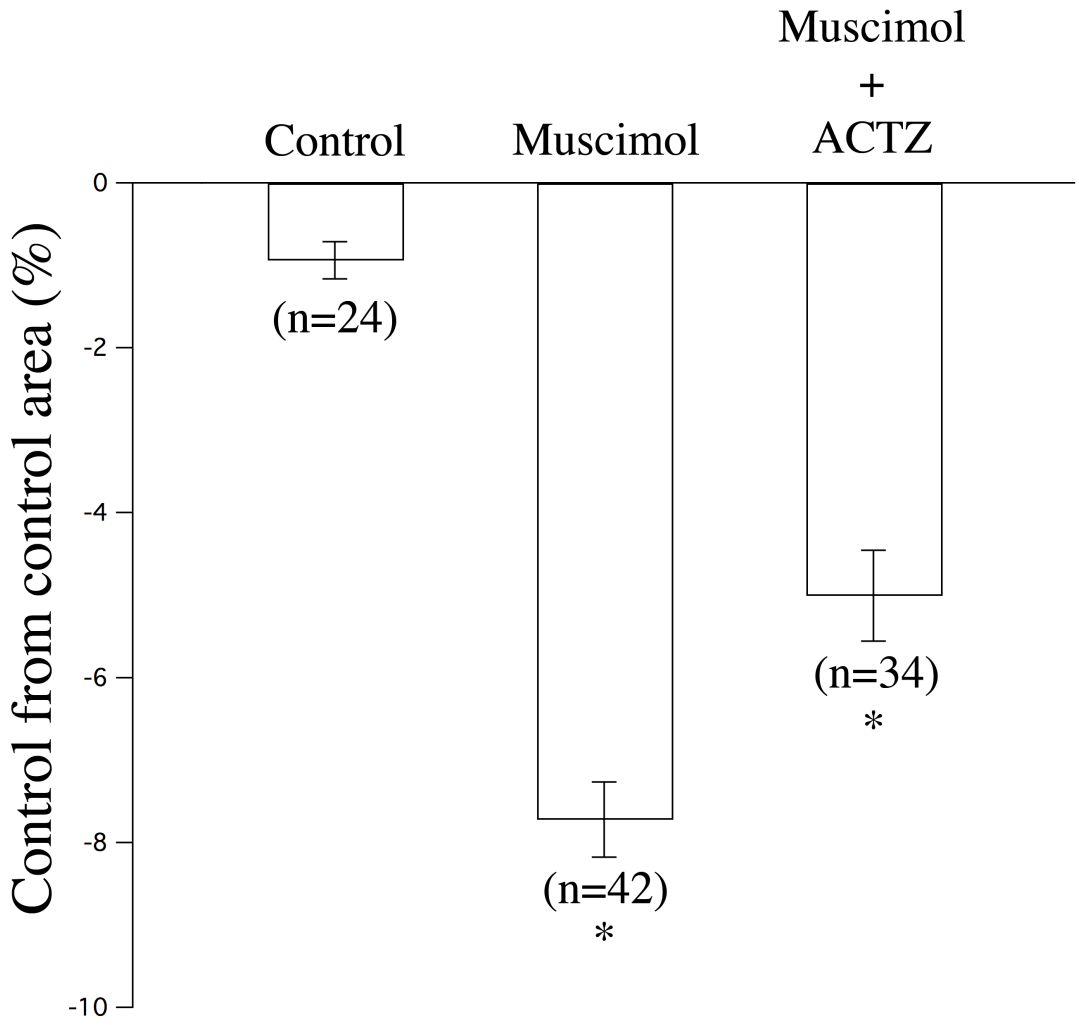

Figure 4.5. HCO_3^- dependent astrocyte shrinkage in response to $GABA_A$ receptor activation.
Astrocyte somas and primary processes shrink in response $GABA_A$ receptor activation with muscimol. The $GABA_A$ receptor mediated astrocyte shrinkage is significantly reduced by inhibiting the production of intracellular HCO_3^- by the membrane-permeant CA inhibitor ACTZ . Bar graph shows the percent changes in the area of the astrocyte somas and primary processes in control ACSF, in ACSF containing the selective $GABA_A$ receptor agonist muscimol, and in ACSF containing muscimol and ACTZ. Error bars denote the SEM.
* Statistical significance assessed using a one-way ANOVA followed by a Bonferroni post hoc analysis, $p<0.05$.

4.4 Neuronal activity induces astrocyte swelling

To determine whether astrocyte volume changes occur as a result of neuronal activity, the volume of astrocytes was quantified during stimulation of the CA3 – CA1 Schaffer collateral fiber pathway in the hippocampus. Stimulation (5 Hz, 4 minutes) induced rapid astrocyte swelling that reached a maximum (4.0 ± 0.4%; n=13 cells from 6 slices) at 1 minute and returned to baseline within 4 minutes (Figure 4.10). To examine if HCO_3^- played a role in the stimulation evoked astrocyte swelling, astrocyte volume was quantified during Schaffer pathway stimulation in hippocampal slices incubated and perfused in HEPES buffered HCO_3^- free ACSF.

In the absence of HCO_3^-, the activity induced astrocyte swelling was reduced to 1.5± 0.2% (n=14 cells from 6 slices), representing a significant decrease in astrocyte swelling compared to astrocytes in control ACSF [$t(25)=5.78$, $p<0.05$; Figure 4.6 A, B).

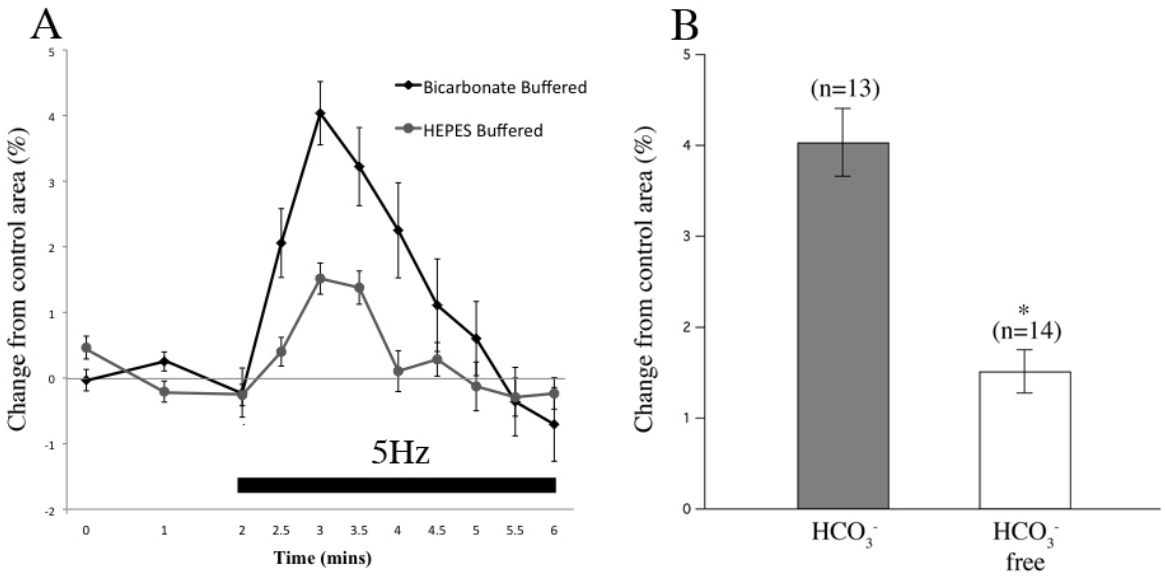

Figure 4.6. Schaffer collateral stimulation causes astrocyte swelling that is dependent on HCO_3^-.
A) Graph of the time-course of the activity induced astrocyte swelling in HCO_3^- buffered ACSF and HEPES buffered HCO_3^- free ACSF. Astrocyte swelling was found to reach a maximum after 1 minute of stimulation (5 Hz, 4 minutes) before decreasing back to baseline volume despite the continued stimulation. Black bar represents the Schaffer collateral stimulation. **B)** Bar graph shows the percent changes in the area of the astrocyte somas and primary processes that result from Schaffer collateral stimulation (imaged at 1-minute time point) in HCO_3^- ACSF (shaded) and HEPES buffered HCO_3^- free ACSF (no fill). Error bars denote the SEM.
* Statistical significance assessed using a Student's t-test, $p<0.05$.

CHAPTER 5

GENERAL DISCUSSION

In the present study, real-time two-photon imaging was used to directly examine astrocyte volume changes and their underlying mechanisms. Astrocytes were found to swell in response to a physiological increase in extracellular K^+, and to shrink in response to $GABA_A$ receptor activation. It was found that both the astrocyte swelling and shrinking appear to be regulated in a HCO_3^- dependent manner. Furthermore, the existence of rapid astrocyte volume changes in response to neuronal activity was confirmed.

5.1 Astrocyte swelling in response to physiological increases in $[K^+]_o$

Local changes in $[K^+]_o$ occur in the brain as a direct result of neural activity. For normal neuronal functioning to be maintained, these changes in $[K^+]_o$ must be tightly regulated. Astrocytes are thought to play a key role in this regulation by taking up excess K^+ from the ECS (Kofuji and Newman, 2004; Walz, 2000). Previous studies have described astrocyte swelling in conjunction with extracellular K^+ buffering (Su et al., 2002; Sykova et al., 1999; Walz, 1991). However, these studies have always been conducted using large, pathological concentrations of K^+ that are not applicable to studies of normal physiological function. Furthermore, astrocyte swelling in response to a physiological increase in $[K^+]_o$ has never before been measured directly in a brain slice preparation. All previous studies used either cultured astrocytes (Su et al., 2002; Walz, 1991) or immunohistochemical staining of fixed tissue (Sykova et al., 2003) to assess astrocyte volume changes. By contrast, the experiments in this thesis were conducted using a two-photon imaging method that allowed for the astrocytes to be imaged at a greater spatial resolution and in real-time as they underwent volume changes. Using this method to directly measure astrocyte volume changes, it was found that a small increase in $[K^+]_o$ evokes a significant swelling of the soma and primary processes of hippocampal astrocytes. Gradual cellular swelling has been reported in brain slices when they are incubated in ACSF (MacGregor et al., 2001; Siklos et al., 1997). However this did not appear to be a factor in the present study; control astrocytes that were incubated continuously in the low K^+ showed no indication of swelling over a 40-minute period.

Cell culture studies have previously found that astrocytes swell in response to glutamate (Bender et al., 1998; Hansson and Ronnback, 1992). It was possible that swelling observed in response to the increased $[K^+]_o$ was occurring in response to increased glutamate release due to an increase in neuronal excitability. However, the effect of increased neuronal excitability appeared to be minimal in this case, as the inhibition of neuronal activity with TTX reduced the swelling by only a small amount.

The ability of astrocytes to buffer K^+ from the ECS is dependent, in part, on K^+ uptake through K_{ir} channels (D'Ambrosio et al., 2002; Djukic et al., 2007; Olsen and Sontheimer, 2008). Because of the involvement of K_{ir}s in K^+ uptake, the influx of K^+ through these channels is one proposed mechanism by which extracellular K^+ may cause astrocyte swelling (Otsby et al., 2009). In accordance with this, in this thesis it was found that K_{ir} channel inhibition significant decreases K^+ induced astrocyte swelling. However, a large portion of the swelling remained in the absence of K_{ir} channel activity, indicating that these channels are not the sole mediators of astrocyte K^+ uptake. Other K^+ uptake mechanisms, such as the Na^+-K^+ ATPase and NKCC1 (MacVicar et al., 2002; Simard and Nedergaard, 2004), are likely involved.

Astrocytes rapidly undergo an intracellular alkalinization in response to increases in $[K^+]_o$ (Pappas and Ransom, 1994; Dietmer and Rose, 1996). This alkalinization is dependent on the uptake of HCO_3^-, and can be blocked with the removal of extracellular HCO_3^- or by inhibition of the main astrocyte HCO_3^- transporter, the NBC (Nagelhus et al., 2004; Obara et al., 2008), is inhibited using DIDS (Brookes and Turner, 1994; Chesler and Craig, 1987; Pappas and Ransom, 1994). Though this alkalinizing effect of $[K^+]_o$ on astrocytes is known, the involvement of HCO_3^- in K^+ induced astrocyte swelling was previously only speculated (Otsby et al., 2009; Walz, 1991). In the present study, the removal of HCO_3^- from the extracellular solution resulted in a significant decrease in the magnitude of the K^+ induced swelling. The dependence of the swelling on extracellular HCO_3^- was further confirmed with the finding that the inhibition of extracellular CA activity also significantly reduced the magnitude of the swelling. Furthermore, the K^+ induced swelling was found to be significantly decreased in the absence of NBC activity, confirming the NBC as the most likely contributor to the HCO_3^- uptake.

The above findings support a mechanism for K^+ induced astrocyte swelling that was first proposed by Nagelhus and colleagues (2004). These authors suggested that an increase in the $[K^+]_o$ as a result of neuronal activity causes a net driving force for K^+ influx into astrocytes while

at the same time causing a depolarization of the astrocyte membrane. This depolarization is believed to activate the uptake of Na^+ and HCO_3^- through the NBC. The resulting accumulation of K^+, Na^+, and HCO_3^- in the astrocyte cytoplasm would generate an osmotic gradient and drive water influx into the astrocyte (Figure 5.1). Interestingly, this mechanism would serve to clear both the ionic bi-product, K^+, and the metabolic bi-product, CO_2, of neuronal activity from the ECS at the same time (Nagelhus et al., 2004).

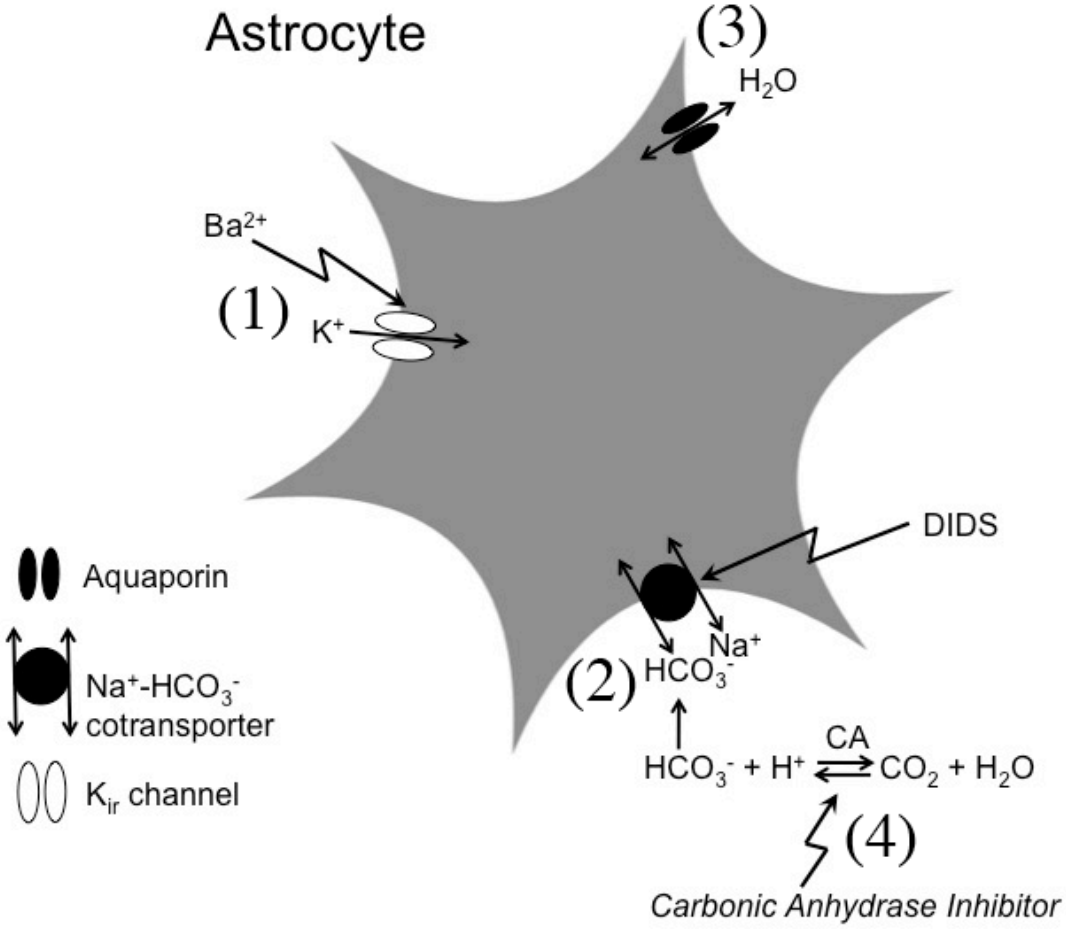

Figure 5.1. A diagram depicting the mechanism of K^+ induced astrocyte swelling.
(1) Extracellular K^+ accumulation stimulates depolarization of the astrocyte membrane and K^+ entry via K_{ir} channels. K_{ir} channels can be blocked by the addition of Ba^{2+} to the bath solution. (2) A depolarization of the astrocyte membrane stimulates the electrogenic NBC to pump Na^+ and HCO_3^- into the cell. The NBC can be blocked by the addition of DIDS to the bath solution. (3) The accumulation of solutes inside the cell drives water inwards causing the astrocyte to swell. (4) The formation of HCO_3^- in the extracellular solution can be blocked by the addition of a membrane impermeant CA inhibitor.

5.2 $GABA_A$ receptor mediated astrocyte shrinkage

Astrocyte $GABA_A$ receptor activation has been shown to induce an acidification of the astrocyte cytoplasm (Chen and Chesler 1992; Kaila et al., 1991). Because this acidification is blocked by the removal of HCO_3^- from the system, it is believed to occur as a result of net HCO_3^- efflux through the $GABA_A$ receptor coupled Cl^- channels (Bevensee et al., 1997; Chen and Chesler, 1992; Kaila et al., 1991). Interestingly, given that the influx of HCO_3^- has been linked to astrocyte swelling, the application of $GABA_A$ receptor agonists has previously been shown to have no effect of the volume of cultured astrocytes (Hansson and Ronnback, 1992). In contrast to the findings of Hansson and Ronnback (1992), the results of this thesis clearly demonstrate that $GABA_A$ receptor activation induces a significant decrease in the volume of hippocampal astrocytes. Furthermore, this shrinkage was significantly decreased when the formation of intracellular HCO_3^- was blocked prior to $GABA_A$ receptor activation.

As discussed in the previous section, the discrepancy between the results of this thesis and previous studies is likely due to differences in the methods used for cell volume measurements. In their study, Hansson and Ronnback (1992) employed a radioactive protein assay method to calculate the intracellular water content of their cultured astrocytes. This indirect method of volume measurement is vastly different from the more sensitive real-time imaging method used in this thesis to directly measure astrocyte swelling.

Because astrocytes actively accumulate Cl^-, the opening of $GABA_A$ receptor coupled Cl^- channels in astrocytes results in an efflux of Cl^- and HCO_3^- (Walz, 2002). It is possible that the $GABA_A$ receptor-mediated astrocyte shrinkage was occurring due to the efflux of Cl^- instead of HCO_3^- from the astrocyte cytoplasm. However, in support of the role for HCO_3^- in astrocyte volume changes, Dr. Sean Mulligan has found that the activation of $GABA_A$ receptors also causes neuronal shrinkage (unpublished results). This shrinkage occurs despite the fact that, unlike in astrocytes, the opening of these channels in neurons results in an influx of Cl^- in addition to the HCO_3^- efflux (Bekar and Walz, 1999; Staley et al., 1995). Therefore, it would appear that in response to $GABA_A$ receptor activation, changes in the volume of astrocytes and neurons are likely driven by the direction of HCO_3^-, and not Cl^-, movement. Taken together, this finding, in addition to the results of the K^+ induced swelling experiments, provides evidence for a HCO_3^- dependent mechanism in astrocyte volume changes.

5.3 Astrocyte swelling in response to neuronal activity

In brain slices, changes in the ECS and optical properties of the tissue have been observed in response to neuronal stimulation (Holthoff and Witte, 1996; MacVicar and Hochman, 1991; Otsby et al., 2009; Sykova et al., 2003). Because astrocytes are the type of brain cells with the highest propensity for swelling, the tissue changes that occur in response to neuronal activity are assumed to occur as a result of glial swelling (MacVicar and Hochman, 1991). However, astrocyte swelling in response to neuronal activity has never before been shown directly. In the present thesis, stimulation of neuronal activity in the Schaffer collateral fiber pathway resulted in swelling of astrocytes within the activated area. As further confirmation for the role of HCO_3^- in astrocyte volume changes, the activity induced swelling was significantly decreased if the stimulation was performed on hippocampal slices that were incubated in HEPES buffered, HCO_3^- free ACSF.

It is important to note that maximum swelling observed in response to neuronal stimulation was much smaller than the swelling observed in response to the 3 mM increase in $[K^+]_o$. This finding is interesting given that neuronal stimulation can reportedly raise the $[K^+]_o$ by as much as several millimolar (Ransom et al., 2000; Olsen and Sontheimer, 2008). It is possible that neuronal stimulation induces a non-uniform increase in the $[K^+]_o$ that does not completely encompass all sides of the astrocytes. A non-uniform increase in $[K^+]_o$ would likely induce less swelling than a uniform increase, such as would be the case with the bath application of the increased K^+ ACSF, surrounding the entire astrocyte. Additionally, long periods of exposure to increased levels of extracellular K^+ have previously been shown to induce an upregulation of GFAP in astrocytes (Sykova et al., 2003). The upregulation of this structural filament may account for the larger magnitude of the astrocyte swelling observed in response to the bath applied increase in $[K^+]_o$.

5.4 Implications for brain functioning

Perhaps the most obvious implication of astrocyte volume changes is their effect on the ECS (Kimelberg, 2000). Because they possess large numbers of highly branched processes, astrocytes constitute a major barrier to diffusion in the brain (Theodosis et al., 2008; Sykova and Chvatal, 2000). Changes in the placement or the volume of the astrocytes, such as was demonstrated in this thesis, may have a significant effect on the ability of substances to diffuse

within the ECS (Sykova et al., 1999). In addition to this, because astrocyte swelling involves the removal of water from the ECS, the concentration of local solutes, such as ions and signaling molecules, may be increased (Kimelberg, 2000). Since extrasynaptic volume transmission constitutes a major signaling mechanism between brain cells (Agnati et al., 1995; Chvatal et al., 2004; Sykova and Chvatal, 2000), any changes in the diffusion properties of the ECS have the potential to greatly affect brain cell signaling (Kimelberg et al., 2000).

The intimate anatomical relationship between fine astrocyte processes and synapses in the brain facilitates the involvement of astrocytes in the regulation of synaptic plasticity (Panatier and Oliet, 2006). Astrocyte endfeet contain high numbers of glutamate transporters (Chaudhry et al., 1995; Lehre et al., 1995), which are essential for glutamate uptake from the synapse and the maintenance of low extracellular glutamate concentrations (Bergles and Jahr, 1998). Upon release from an active excitatory synapse, glutamate can diffuse out of the synaptic cleft to stimulate extrasynaptic receptors and neighbouring synapses through a phenomenon known as "spillover" (Diamond, 2001; Wong et al., 2007). In the hippocampus, glutamate spillover, and therefore the activation of extrasynaptic glutamate receptors, is limited by the activity of glutamate transporters on the surrounding astrocytes (Asztely et al., 1997; Diamond, 2001). Due to their involvement in glutamate uptake, astrocytes may be directly involved in the induction of one form of synaptic plasticity, long-term depression (LTD), which appears to occur when glutamate spillover from the synapse activates extrasynaptic NMDA receptors (Massey et al., 2004; Wong et al., 2007). The induction of LTD is facilitated when astrocyte glutamate uptake is blocked to allow for a greater amount of glutamate spillover from the synapse (Massey et al., 2004; Wong et al., 2007).

One physiological mechanism that might contribute to a decreased ability of astrocytes to clear glutamate from around the synapse might be an increase in the distance between the astrocyte processes and the synapse. The effect that the distance between astrocyte processes and the synapse has on synaptic transmission has previously been demonstrated in the hypothalamus (Theodosis et al., 2008). In the hypothalamus, reversible retraction of astrocyte processes occurs during times of lactation and dehydration (Panatier and Oliet, 2006). As the processes retract further from the synapse, glutamate clearance from the ECS is delayed and the efficacy of surrounding synapses can be decreased (Panatier and Oliet, 2006; Theodosis et al., 2008; Wang and Hamilton, 2009). The volume changes that were reported in this thesis occur

over a much shorter time period, and on a much smaller scale, than astrocyte process retraction in the hypothalamus. However, the experiments in the hypothalamus clearly demonstrate the importance that the proximity of astrocyte processes to the synapse plays in their ability to regulate synaptic function. Astrocyte swelling in response to extracellular K^+, or shrinking in response to $GABA_A$ receptor activation, is likely not be localized to the soma, but instead may extend to the furthest reaches of the astrocyte processes. In this manner, the volume changes observed in this thesis may play an integral role in the regulation of synaptic transmission over a much shorter time span than what has previously been observed in the hypothalamus.

5.5 Limitations

5.5.1 The brain slice preparation

Brain slices are one of the most widely used *in vitro* preparations for the study of cells in the CNS (Reid et al., 1988). While this preparation offers a number of obvious advantages, such as the ease with which the structure of the tissue and cells can be visualized and the control that can be had over the composition of the extracellular environment (Teyler, 1980), it also comes with a number of disadvantages. The most obvious of these disadvantages is the physical damage that occurs to the cut surfaces on the top and the bottom of the slices, and special care must be taken to ensure that all sampled cells are found within the healthy core of tissue that remains between the two damaged layers (Teyler, 1980). Additionally, once removed from the brain, the tissue has a relatively short lifespan of only a few hours and is no longer connected to the greater circuitry of the brain (for a more in-depth review see Reid et al., 1988). However, this preparation has been used with great success for decades in physiological studies and is able to yield cellular images of a quality that is of yet unachievable in an *in vivo* preparation. This is particularly true for the imaging of the cellular structure of areas deep within the brain, such as the hippocampus, which at this time cannot be directly observed *in vivo* due to the limitations of our current imaging systems.

5.5.2 Astrocyte identification

For the purpose of this thesis, astrocytes in the CA1 region of the hippocampus were identified and imaged based on morphological criteria and the loading of the astrocyte specific dye SR101. Unlike in the neocortex, where SR101 is believed to load all protoplasmic

astrocytes (Nimmerjahn et al., 2004), SR101 may load only one of two main astrocyte subtypes in the hippocampus (Kafitz et al., 2008). Of major concern for the present study is that these two astrocyte subpopulations display differences in both their receptor expression and capacity for K^+ uptake (Zhou and Kimelberg, 2000; Zhou and Kimelberg, 2001). It is possible that the results presented in this study reflect only one of those astrocyte subpopulations, as each population may respond differently to both the K^+ and muscimol stimuli. However, a more recent study of the electrophysiological properties of astrocytes *in vivo* suggests that astrocytes in the grey matter of the hippocampus and the cortex reflect a single homogenous population at maturity (Mishima and Hirashi, 2010), increasing the likelihood that the results presented here pertain to all grey matter astrocytes.

5.5.3 Volume measurements

For the purpose of this thesis, volume measurements were taken only from the astrocyte somas and primary processes to avoid any error that might occur as a result of dye leakage from smaller processes. Although a number of small processes could be observed in the area surrounding each of the cells, the stability of the dye loading in these areas was less than that of the soma and large primary processes. However, this implies that volume changes in a significant portion of each astrocyte were not considered in this thesis. For this reason, it is possible that the values presented here may represent either an over or under estimation of the actual changes in astrocyte volume. In addition, the volume measurements presented here are based on the analysis of maximum intensity projections of 3D image stacks. This method of measurement assumes that changes in the lateral dimensions of the astrocytes are an adequate representation of a uniform volume change that is occurring in all directions around the astrocyte soma. The true cellular volume change is believed to be underestimated with this method of measurement (Risher et al., 2009).

5.5.4 Pharmacological effects

The use of drugs as tools to evaluate physiological processes is extremely common. Although their application can be useful and informative in the study of physiology, many drugs can act on a system in a multitude of ways. In this thesis, several different drugs were applied to the hippocampal slices. While some, like TTX, have a highly selective blocking action (Hille,

1968), others may act upon a number of different targets. For example, DIDS, the NBC inhibitor, also blocks the activity of other HCO_3^- transporters, such as the Na^+-dependent Cl^--HCO_3^- exchanger (Pappas and Ransom, 1994). However, in astrocytes the most prevalent type of the DIDS sensitive transporters is the NBC (Schmitt et al., 2000), and it is therefore likely that the decrease in swelling observed upon DIDS application was due primarily to NBC inhibition. In addition, the expression of HCO_3^- transporters, and $GABA_A$ receptors, and CA is not limited to astrocytes, but is also shared with the surrounding neurons. As such, the possibility that the application of these drugs might alter the system in some unanticipated way through interactions with other cell types should not be discounted.

5.6 Future directions

As previously discussed, astrocyte swelling has the potential to affect not only the size of the ECS, but also the diffusion of substances within it. The application of large, pathological increases in the $[K^+]_o$ results in decreased diffusion of molecules in the ECS (Sykova et al., 2003). In light of this, it would be interesting to assess the impact of the physiologically induced volume changes described in this thesis on the size and diffusion parameters of the ECS.

Preliminary experiments suggest that the astrocyte swelling induced by physiological increases in the $[K^+]_o$ does have an effect of the volume of the ECS. An increase in the $[K^+]_o$ of 3 mM resulted in an increase in light transmittance (an indication of cellular swelling) and a decrease in the amount of fluorescence emitted from a membrane impermeant dye in the ECS (Figure 5.2). Some further efforts were made to study this effect, but the results remain inconclusive and deserve further attention.

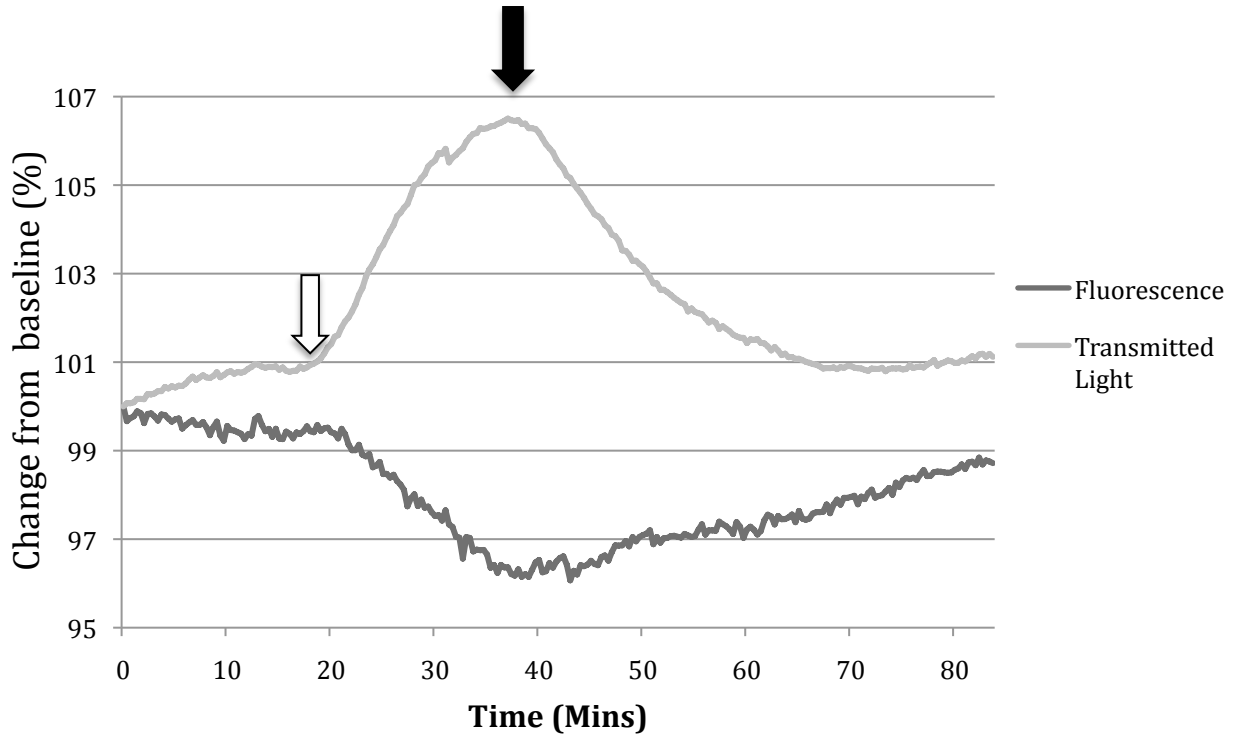

Figure 5.2. Changes in the optical properties of the tissue and the volume of the extracellular space in response to a 3 mM increase in $[K^+]_o$.
Shortly after the application of the high K^+ solution (white arrow) an increase in the transmitted light, and a decrease in the extracellular fluorescence of a 10000 MW Texas Red dextran occurs. This change is reversed when the slice is returned to the low K^+ solution after 20 minutes (black arrow).

CHAPTER 6

CONCLUSION

The results of this thesis demonstrate the existence of astrocyte volume changes in response to physiological increases in the $[K^+]_o$ and to $GABA_A$ receptor activation. Both the K^+ induced swelling and the $GABA_A$ receptor mediated shrinkage were found to be regulated in a HCO_3^- dependent manner. Furthermore, astrocyte swelling was also observed in response to neuronal activity. Although activity-induced astrocyte swelling has been widely assumed to occur, it has never before been shown directly. These volume changes may play a fundamental role in the control of extracellular brain signaling and the regulation of synaptic plasticity.

CHAPTER 7

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